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It is characteristic of Science and Progress that they continually open new fields to our vision.—Pasteur.

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STUDIES ON THE CLASSIFICATION OF THE COLON-TYPHOID GROUP OF BACTERIA WITH SPECIAL REFERENCE TO THEIR FERMENTATIVE REACTIONS

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INTRODUCTION

In recognition of the urgent need of progress along the lines of bacterial classification, the Society of American Bacteriologists at its meeting in Urbana appointed a special Committee on the Characterization and Classification of Bacterial Types. This Committee conceived its first duty to be a general revision of the families and genera of the Bacteriaceae, which was presented to the Society in preliminary form in 1917. In addition to this broad survey of the entire field the Committee projected a more intensive study of the colon-typhoid group, a group which, together with certain sharply defined species, includes many puzzling intermediate forms, difficult of classification, and yet of fundamental medical and sanitary importance. War work and other demands upon the time of the Committee have delayed the completion of this latter task, but certain members of the Committee, particularly Dr. Charles Krumwiede and Dr. L. A. Rogers have already published important individual contributions to the subject (Krumwiede, Pratt and Kohn, 1916a, 1916b, 1917; Krumwiede, Kohn and Valentine, 1918; Rogers, Clark and Evans, 1914, 1915; Rogers, Clark and Lubs, 1918). The present paper is the result of a detailed study of 160 cultures of colon-typhoid bacteria in the collection of the American

Museum of Natural History, conducted in large measure by the application of a series of standardized tests agreed upon by the Committee on Characterization and Classification of Bacterial Types. The laboratory work was for the most part carried out in 1915, 1916 and 1917 and preliminary reports upon its progress were made before the Society of American Bacteriologists in 1915 and 1916 (Winslow and Kligler, 1916; Winslow, Kligler and Rothberg, 1917). Final publication of the results has been delayed until time was available for a careful digestion of the data and a review of the literature concerning the classification of this group of bacteria.

THE MAJOR SUBDIVISIONS OF THE COLON-TYPHOID GROUP

Primary division on the basis of lactose fermentation. It seems reasonably certain that the typhoid bacillus was recognized in stained preparations by Eberth in 1880 and by Koch in the same year, and that Friedlaender saw *Bacterium pneumoniae* in 1882. General interest in this group of organisms dates however from the cultivation of the typhoid bacillus by Gaffky in 1884. This author described the organism as a motile rod, taking up anilin stains less readily than most bacteria, which failed to liquefy gelatin and gave a characteristic growth on potato and blood serum. Either from the presence of contaminating organisms, or a misinterpretation of irregular staining, he reported that it formed spores upon potato at 37°C.

In the following year Escherich (1885) described and figured *Bacterium coli* as a Gram-negative organism producing characteristic colonies and coagulating milk and forming gas in glucose broth. For the next decade attention was largely devoted to the establishment of distinctions between the types described respectively by Gaffky and by Escherich.

This differentiation was naturally based first of all on the production of gas in glucose media (facilitated by Smith's introduction of the fermentation tube in 1889) and on the formation of acid in lactose media. Petruschky (1889–1890) was probably the first investigator to introduce quantitative measurements of

acid production. He cultivated his bacteria in litmus milk and determined the amount of alkali necessary to produce neutrality. *B. suipestifer* gave an alkaline reaction, while the acidity produced by the other organisms studied was as follows: *B. typhosus* 0.2 to 0.3 per cent; *B. pneumoniae*, 0.3 to 0.4 per cent; *B. neapolitanus*, 0.7 to 0.8 per cent; *B. acidi-lactici*, 1.7 to 1.8 per cent. Chantemesse and Widal (1887) and Smith (1890) also pointed out that *B. typhosus* could be distinguished from *B. coli* by its less active fermentation of lactose. Chantemesse and Widal (1891), in controverting the claim of certain workers that *B. coli* and *B. typhosus* were identical, laid great stress on fermentation of sugars as a differential test and suggested the use of sugar broth containing calcium carbonate. They state that *B. coli* forms gas from glucose, sucrose, lactose, maltose, rhamnose, glycerol, erythritol and mannitol, but not from starch or glycogen; and they maintain that *B. typhosus* forms gas from none of these substances. Wurtz (1892) recommended the addition of an indicator to liquid or solid media for the easy separation of the two forms. Germano and Maurea (1893) considered the presence or absence of gas in 2 per cent glucose agar as the one absolutely diagnostic test.

In 1896 Capaldi and Proskauer suggested the use of two special media for the differentiation of the typhoid and colon groups. In medium I, containing 0.2 per cent asparagin and 0.2 per cent mannitol with the necessary salts, *B. typhosus* fails to grow and *B. coli* forms acid, while in medium II, containing 2.0 per cent peptone and 0.1 per cent mannitol, *B. typhosus* forms acid and *B. coli* does not. Medium I of course depends for its differential action on the inability of *B. typhosus* to utilize asparagin as a source of nitrogen, while the difference in the behavior of *B. coli* in the two solutions is presumably related to the balance of nitrogenous and carbohydrate foodstuffs provided. Durham (1901) urged the value of litmus whey as a test medium. Drigalski and Conradi (1902) reported that *B. coli* fermented arabinose and rhamnose while *B. typhosus* failed to do so; and they suggested a new medium for the differentiation of colon and typhoid colonies which, like the ordinary

lactose agar and the Endo (1904) medium, depends on the power of the former bacterium to produce acid from lactose. Meillère (1907) claimed that *B. typhosus* will attack inosite under aerobic conditions, while *B. coli* cannot ferment this substance.

Meanwhile many other distinctions between the typhoid and colon organisms had been described by various observers. Fremlin in 1893 differentiated *B. coli* from *B. typhosus* by its less active motility, fewer and less easily stained flagella, less tendency to occur in chains, more rapid and vigorous growth on various media (including the classical visible yellowish growth on potato), and the power to produce indol—as well as by the coagulation of milk and the formation of gas in glucose media. The active motility of the typhoid organism has since been made a basis for the selective media of Hiss (1902) and Hesse (Jackson and Melia, 1909), and for the isolation methods of Drigalski (1906) and Starkey (1906).

A new differential method of great importance was introduced in 1896 in the agglutination tests of Pfeiffer and Kolle and of Widal; and this test, too, quickly demonstrated the fundamental differences between the colon and typhoid organisms.

The more vigorous reducing power of the colon bacillus is another characteristic, which has been widely used in the form of the nitrate test. The greater ability of the colon bacillus to reduce dyes was suggested as a basis of differentiation by Dunbar (1892) using litmus, and by Rothberger (1898) using neutral red. Lead acetate on the other hand is turned brown by *B. typhosus* and not by *B. coli*, as pointed out by Orlowski, Sacquépée and Chevrel (1905) and Burnet and Weissenbach (1915).

Finally the attempt to devise methods for the isolation of the typhoid bacillus has led to the recognition of a number of more or less specific differences in the tolerance of *B. typhosus* and *B. coli* toward a wide variety of antiseptic substances. The acid broths used for preliminary enrichment in the attempt to isolate the typhoid bacillus from water were designed merely to inhibit the ordinary water bacteria; but Hankin (1899) pointed out that within the colon-typhoid group *B. coli* could bear a

higher concentration of acid than *B. typhosus*. Winslow and Lochridge (1902) showed that the toxic action of the mineral acids was due to their free hydrogen ions and presented the following table of the comparative resistance of the colon and typhoid organisms.

Disinfectant action of mineral acids

	PARTS OF DISSOCIATED HYDROGEN PER MILLION NECESSARY TO EFFECT			
	99 per cent reduction		100 per cent reduction	
	HCl	H_2SO_4	HCl	H_2SO_4
B. typhosus	2.94	2.54	4.85	3.90
B. coli	7.49	7.68	12.80	12.60

Loeffler (1903, 1906) and Lentz and Tietz (1903, 1905) reported that *B. typhosus* was more resistant than *B. coli* to the action of malachite green. A whole group of green dyes seem to be favorable for the selective cultivation of the typhoid bacillus and its allies. Werbitzki employed China green for this purpose and Conradi, brilliant green. The whole group of the green dyes has been studied very carefully by Krumwiede and Pratt (1914). They found brilliant green most satisfactory for this purpose, the paratyphoid-enteritidis forms being most resistant, *B. typhosus* coming next, and the colon-aerogenes group being most readily inhibited. Roth (1903) and Hoffman and Ficker (1904) maintained that *B. typhosus* was more resistant to caffeine than the colon bacillus. Nowack (1905) thought that a slightly alkaline reaction exerted a selective influence on the typhoid organism. Jackson and Melia (1909) found *B. typhosus* more resistant to the action of bile salts than *B. coli*.

Davis (1914) has studied the resistance of the members of the colon-typhoid group to potassium tellurite, and finds that the colon-aerogenes forms are most resistant, followed in succession by the paratyphoid B and Gaertner group, *B. typhosus*, *B. paratyphosus* A and the dysenteries. Capsulated organisms vary widely in susceptibility, *B. capsulatus* appearing at the top and *B. pneumoniae* and *B. rhinoscleromatis* nearly at the bottom of

the list. *B. acidi-lactici* somewhat surprisingly proves one of the sensitive organisms, while *B. zopfii* is most susceptible of all.

Manfredi (1917) reports that a concentration of cholesterin of 0.5 per cent will check typhoid and the paratyphoids, while *B. coli* will grow in concentrations up to 1 per cent.

During the decade following the first cultivation of the typhoid and colon bacilli it was made clear that these organisms belong to a large group of Gram-negative non-spore-forming bacilli. They are plump straight rods with rounded ends which grow under both aerobic and anaerobic conditions on ordinary media producing colonies which vary from a thin translucent irregularly notched "grape-leaf" form to a more compact and opaque, very slightly yellowish, colony, and possessing more or less marked powers of decomposing carbohydrate materials.[1]

This main group may be sharply split into two primary subdivisions by the presence or absence of the power of fermenting lactose. The lactose fermenters are generally more vigorous in their attack on other carbohydrates as well. They are less actively motile. They grow more abundantly on media and exert a more powerful reducing action. They generally produce indol. They are as a rule of low pathogenic power. The non-lactose-fermenters differ in all of the characteristics cited; and each type has its specific agglutinative reactions and its more or less specific degree of tolerance of various antiseptic substances.

SECONDARY SUBDIVISION OF THE NON-LACTOSE FERMENTERS

Considering next the further subdivision of the typhoid or non-lactose-fermenting series, the first important step was the discovery that certain members of this series possessed the power of forming gas in glucose media. *B. suipestifer* discovered

[1] Our whole conception of the morphology of this group of organisms will be radically altered if the studies of Hort (1917) are confirmed. This investigator describes in acid broth (and to some extent in ordinary broth) cultures of typhoid, paratyphoid, dysentery and colon organisms extraordinary reproductive stages, characterized by budding, terminal, lateral, or superficial, with the production of cruciform and radial cell aggregates, and he believes that detached buds may constitute a filterable stage in the life cycle of the organisms.

by Salmon and Smith in 1885 and *B. enteritidis*, demonstrated as the cause of an outbreak of meat poisoning by Gaertner in 1888, were the first organisms of this type to attract attention. Smith (1893a) pointed out that *B. suipestifer* produced gas in glucose but not in lactose media, and Durham (1898) noted that the Gaertner bacillus differs from the typhoid bacillus, not only in its power to form gas but in the lower final acidity in lactose media. The table below presented by him is an exceedingly interesting one as a product of a period when quantitative methods were so rarely used in bacteriological investigations.

Acidity, per cent normal acid

MEDIUM	PERIOD (AT 37°C.)	B. TYPHOSUS	B. ENTERITIDIS	B. COLI
Neutral	24 hours	0.25–0.35	0.2–0.3	0.6–0.8
Litmus whey	4–5 days	Less than 0.6	–0.15	Over 1
Litmus medium	24 hours	0.15	0.3	0.5
Litmus medium containing 2 per cent peptone	Several days	0.6	0.35	–0.2
Litmus medium containing 1 per cent glucose	Eventually	Generally alkaline	Alkaline	Alkaline

In a later communication Durham (1901) shows that litmus whey is of special value for differentiating these forms, *B. enteritidis* producing like *B. typhosus* an initial acid reaction, but unlike *B. typhosus* causing a subsequent change to alkalinity.

Meanwhile other types of colon-typhoid intermediates were being reported from various sources. Gwyn in 1898 described a case of a disease clinically like typhoid fever from which he isolated an organism resembling *B. enteritidis*, which be called a "paracolon" form (using a term first introduced by Gilbert in 1895). Cushing (1900) described a similar organism, which he called Bacillus 0, and like Durham clearly recognized the existence of a series of intermediate forms, resembling *B. typhosus* in morphology and motility, in the possession of pathogenic power, in failing to ferment lactose or produce indol, and in

relatively meager growth, but resembling *B. coli* in producing gas in glucose and differing from both in producing a transient acid reaction in milk with later reversion to alkalinity.

In 1901 Schottmüller published his classical paper on the paratyphoids. Six different strains of intermediates were worked out in detail. It was shown that these forms like those studied by Durham, Gwyn and Cushing were characterized by active motility, growth intermediate in luxuriance between that of *B. coli* and that of *B. typhosus*, gas production in glucose media, and reduction of neutral red. From the typhoid bacillus they were also distinguished by colony structure on Piorkowski's alkaline urea-gelatin, by the decolorization of an indigo compound and by a peculiar transparency in milk due to alkali production. In litmus-whey he distinguished two types of the "intermediates," one maintaining an acid reaction, the other changing to a blue-violet color. These forms, which we now know as the paratyphoids A and B, were also differentiated by their growth on media and their agglutination reactions.

By the early nineties then the non-lactose-fermenters had been separated into two clearly defined subgroups which may most conveniently be distinguished by the presence or absence of the power to produce gas in glucose media.

FURTHER SUBDIVISION OF THE NON-LACTOSE FERMENTING ORGANISMS

Each of the two groups, to which reference has been made above, was further subdivided during the early years of the twentieth century.

The first step in the differentiation of types among the bacilli which fail to form gas in either glucose or lactose was the discovery of the bacillus of dysentery by Shiga in 1898. Similar organisms were isolated by Kruse in 1900 and by Flexner and by Strong and Musgrave in the same year. Flexner (1901) distinguished the dysentery from the typhoid organisms by less active motility, general tendency to produce indol, secondary alkaline reaction in milk, and characteristic agglutination reac-

tions. After a long controversy as to the specificity of the various dysentery organisms the investigations of Martine and Lentz (1902), Duval and Bassett (1902), Hiss and Russell (1903), Park, Collins and Goodwin (1903) and others made it clear that there were at least two quite distinct types of dysentery bacilli, the Shiga type which ferments only the monosaccharides and forms no indol and a second type including the Flexner, Strong and Hiss strains which produces indol and ferments both glucose and mannitol and in some cases other carbohydrates as well. The Flexner types are most nearly allied by their fermentative reactions to *B. typhosus*, while the Shiga strains represent a group of exceedingly limited metabolic powers. J. H. Smith (1915) is, we believe, correct in using the fermentation of mannitol for the primary classification of the forms which produce no gas in glucose, and we shall follow him in recognizing as the first two subdivisions of the colon-typhoid series:

Group I. Organisms fermenting no carbohydrates except the simple hexoses (including here for convenience *B. alcaligenes* as well as the Shiga type of dysentery).

Group II. Organisms fermenting the hexoses and mannitol and certain other carbohydrates; forming only acid but no gas (including *B. typhosus* and the Flexner and other mannitol fermenting types of dysentery bacilli).

Turning now to the paratyphoids themselves, the organisms which ferment glucose with gas production but fail to attack lactose, it will be remembered that Schottmüller even in 1900 recognized two distinct types of paratyphoid organisms according to their reaction in litmus milk. Kayser (1904) emphasized this distinction more clearly and showed that Paratyphoid B resembled most closely the organisms of the Gaertner food poisoning group. Morgan (1905) studied 21 B strains and 10 A strains, the former producing a permanent acid reaction in milk and forming indol, while the latter turned the milk first acid and then alkaline, failed to form indol and gave agglutinative reactions with sera of the *B. enteritidis* group. Boycott (1906) was perhaps the first investigator to point out that even with the A strains milk cultures eventually became alkaline. This

conclusion was confirmed by Bradley (1912), Krumwiede, Pratt and Kohn (1916b), Hadley (1917) and others, and Jordan (1918) shows that the difference between the two types is probably due merely to a difference in their rate of multiplication. Buxton (1902), Bainbridge (1909) and others emphasize the less vigorous action of the A strains in glucose media, and point out that with neutral red these organisms often fail to show reduction, which is always present with the B strains. Buxton noted the peculiar opalescence in milk cultures of the B organisms and attributed it to a solution of casein caused either by the alkalinity or by the presence of a casease.

Aside from the quantitative difference in their action upon milk, the A and B divisions of the paratyphoid series exhibit other distinct differences in fermentative power. Ford (1905) concluded that *B. suipestifer* fermented only rhamnose, mannitol, and dulcitol besides the hexoses, while Schottmüller's paratyphoid A fermented arabinose also and *B. enteritidis* arabinose and xylose. Later investigations indicate that *B. suipestifer* does attack xylose and that dulcitol fermentation is generally absent in the A paratyphoids and *B. suipestifer*. The difference in dulcitol fermentation was noted particularly by Ditthorn (1913). The fact that *B. suipestifer* as well as paratyphoid B and *B. enteritidis* all ferment xylose, while paratyphoid A does not, was clearly shown by Harding and Ostenburg (1912) and Krumwiede, Pratt, and Kohn (1916a). Weiss and Rice (1917) and Hulton-Frankel (1918) point out that fermentation of inosite furnishes another differential test, paratyphoid B attacking this substance while paratyphoid A fails to do so. *B. suipestifer*, and curiously enough *B. enteritidis*, also resemble the A strains in this respect. Exhaustive studies by Krumwiede, Pratt and Kohn (1917), Jordan (1917), and Krumwiede, Kohn and Valentine (1918) have confirmed the fundamental fact that the paratyphoid group may be subdivided in the following manner on the basis of fermentative power.

	XYLOSE	ARABINOSE	DULCITOL	INOSITE
Para A	−	+	±	−
B. suipestifer	+	−	±	−
B. enteritidis	+	+	+	−
Para B	+	+	+	+

Another interesting line of differentiation between the two main subdivisions of the paratyphoid group is the color reaction in media containing lead or iron salts, first suggested by Orlowski. Sacquépée and Chevrel (1905) pointed out that on a gelatin medium containing the double tartrate of iron and potassium, or subacetate of lead, paratyphoid B and *B. typhosus* produce a black coloration (due presumably to precipitation of lead sulphide), while paratyphoid A and *B. coli* fail to do so. Recently Burnet and Weissenbach (1915), Weissenbach (1917), Kligler (1917), and Jordan and Victorson (1917) have confirmed these results, which occur with marked regularity, and the latter investigators show that, as in most respects, *B. suipestifer* behaves like paratyphoid A.

A similar color reaction, suggested by Orlowski, and used by Sacquépée and Chevrel (1905) and Weissenbach (1917), is the production of a green coloration in gelatin containing nitroprussiate of sodium. In such a medium paratyphoid B produces an intense green coloration in forty-eight hours and *B. typhosus* in three days; while the reaction fails entirely with *B. coli* and paratyphoid A.

The various investigations cited make it clear that we may recognize at least two more groups of the non-lactose-fermenters (in addition to those cited on p. 437).

Group III. Organisms fermenting glucose and the other hexoses with the formation of gas; fermenting mannitol and rhamnose and arabinose, in a similar way, but failing to ferment xylose and lactose and usually failing to ferment dulcitol; producing an acid reaction in milk which persists for from five days to six weeks; this group includes the A paratyphoids.

Group IV. Organisms differing from members of group III in fermenting xylose and generally dulcitol; sometimes failing to attack arabinose; producing a transient acid reaction in milk which turns to alkaline in two to five days; this group includes *B. suipestifer*, *B. enteritidis*, paratyphoid B and their allies.

SECONDARY SUBDIVISION OF THE LACTOSE-FERMENTERS

The non-lactose-fermenters may be separated, as indicated above, into four secondary groups which are rather clearly differentiated in each case by definitely correlated bio-chemical properties, as well as characteristic serological and pathogenic relations. The classification of the lactose-fermenting division of the colon-typhoid series has proved a much less simple task.

Escherich (1885) recognized two distinct types of organism in this group, *B. coli-commune* which he described as a Gram-negative rod producing characteristic colonies, gas in glucose media, and slow coagulation of milk, and *B. lactis-aerogenes* which he differentiated by the plumper form of the cells, lack of motility, and more rapid coagulation of milk. These differences seem somewhat slight but they have been shown by later observers to be correlated with much more fundamental characters. Smith (1893a) pointed out the heavier growth and tendency to capsule and zooglea formation on the part of *B. aerogenes*. In a later paper (Smith 1895a) he emphasizes the rapid gas production and relatively low acidity characteristic of this organism, and points out that the ratio of CO_2 to H_2 is higher in some strains of *B. aerogenes* (and in all the strains of *B. cloacae* studied) than is the case with *B. coli*. Russell and Bassett (1899) also laid stress on the difference in the CO_2 to H_2 ratio and suggested that the forms producing the larger proportion of CO_2 were not of intestinal origin, but were perhaps normal soil bacteria. Durham (1901) noted that the *B. aerogenes* types fermented starch and inulin and gave the peculiar reaction described by Voges and Proskauer in 1898. Grimbert and Legros in 1900 and Jordan (1903) pointed out that organisms of the *B. aerogenes* series frequently fail to form indol. A most important step in the

classification of this group was taken when Harden and Walpole (1905), and Rogers, Clark and Davis (1914) accurately measured the ratio of CO_2 to H_2 (which had been roughly estimated in an open fermentation tube by Smith and the other workers cited above); and showed that the colon forms actually produce these two gases in approximately equal proportions, while with the aerogenes type the true ratio of CO_2 to H_2 is about 2 to 1. Clark and Lubs (1915) discovered that the two types may be easily distinguished by a difference in the acid reaction produced in sugar broths of specified composition, and introduced the simple methyl-red test for measuring this difference. These investigators and others to be cited later have made it clear that in a number of respects the two types mentioned are sharply differentiated, so that we may recognize two more distinct groups of the colon-typhoid series, characterized as follows:

Group V. Organisms fermenting the hexoses, mannitol, xylose, rhamnose, arabinose and lactose, frequently salicin, dulcitol, sucrose, raffinose and glycerol; producing a strong acid reaction in media containing these substances and a moderate amount of gas, composed in equal parts of CO_2 and H_2; producing indol and failing to give the Voges-Proskauer reaction; generally motile and not capsulated; never liquefying gelatin.

Group VI. Organisms differing from Group V in producing a lower acidity in carbohydrate media and a larger proportion of gas, of which two thirds is CO_2; in fermenting dulcitol and glycerol less frequently and adonitol, salicin, starch, sucrose, raffinose and inosite more frequently; in giving a generally negative indol and a constantly positive Voges-Proskauer reaction; in being generally non-motile and frequently capsulated; and in sometimes liquefying gelatin.

The distinction between these two groups is a very sharp and clean cut one, as indicated by the large number of correlated characters in which they differ. Lines of demarcation within these groups of the lactose-fermenting organisms are unfortunately much less clear. Smith as early as 1893 recognized that among the low ratio (*B. coli*) strains there are some which attack sucrose and some which do not, and that among the high ratio forms *B. cloacae* (as described by Jordan in 1890) has the

power of effecting a sluggish liquefaction of gelatin. Durham in 1901 limited the term *B. coli-communis* to the sucrose-negative form and named the sucrose-positive form *B. communior*. Winslow and Walker (1907) pointed out that the fermentation of raffinose varies together with that of sucrose; but this is perhaps less a correlated character than an independent measurement of the same character since these two sugars have a similar molecular configuration. As a matter of fact no very striking correlations are apparent between any of the characteristics of these organisms, and the attempts made by MacConkey (1905) Jackson (1911) and others to classify them according to their fermentative reactions are purely arbitrary and artificial. The same thing is true of classifications like those of Ford (1903) and Jordan (1903) in which the liquefaction of gelatin is given an important place. MacConkey (1905) laid special stress upon dulcitol for differential purposes, Kligler (1914) upon salicin and glycerol, while Levine (1917) has made perhaps the most important contribution to the whole problem. Certain types recognized by Levine will be discussed later on, but for the purposes of a general subdivision of the colon-typhoid series it is perhaps only necessary to point out that group V as defined above includes a sucrose-positive and a sucrose-negative subgroup, while group VI includes forms which liquefy gelatin and others which fail to do so.

THE VALUE OF CARBOHYDRATE FERMENTATION TESTS AS A BASIS FOR BACTERIAL CLASSIFICATION

It will be noted that almost all observers who have attempted to differentiate and classify the organisms of the colon-typhoid series have laid primary stress upon the fermentative reactions of these organisms in carbohydrate media, and it is important to know whether such reactions are of sufficiently fundamental biological importance to warrant such an emphasis.

The results obtained by certain investigators have tended to cast some doubt upon the constancy and reliability of fermentation tests as applied to the members of this group. In the first place intermediate forms have been described which differ from typical ones either in exhibiting a slow but ultimately distinct

action upon a particular carbohydrate, or in displaying a combination of fermentative characters the reverse of that which normally occurs. Thus Wilson (1910) describes a curious form isolated from the urine of a carrier which at 37° produced no acid in lactose media and but little gas in mannitol and maltose, while at 20° it formed an abundance of acid in lactose and an abundance of gas in mannitol, maltose and salicin media. Glucose and sucrose always showed acid but no gas. Oette (1913) notes a peculiar strain which possessed the general fermentative reactions of the paratyphoids and showed specific agglutination with paratyphoid B but produced no gas. Raubitschek and Natonek (1913) isolated 31 different strains of typhoid bacilli from the various organs of two patients and found marked quantitative differences in fermentative power between them. Tenbroeck (1916) describes a variant arising in an old culture of *B. suipestifer* which retained all its normal cultural and immunological characteristics except that it no longer produced gas in glucose and failed to reduce neutral red. Smirnow (1916) exposed a series of colon strains to the influence of strong glucose solutions (3 per cent), NaCl (4 per cent), Na_2SO_4 (1.5 per cent) and phenol (0.25 to 0.75 per cent). After successive transfers, covering a period of one to three months, he found a marked suppression of biochemical activities, glucose and phenol exhibiting the most marked effects. Indol formation was first affected, then gas production in various carbohydrate media, then the characteristic growth on potato, then the coagulation of milk, and finally the production of acid in carbohydrate media. The cultures generally reverted to their normal characteristics on prolonged cultivation in ordinary media, but some of the induced modifications proved permanent. Hadley, Caldwell, Elkins and Lambert (1917) recognize two varieties of *B. pullorum*, one of which produces gas while the other fails to do so. Oliver and Perkins (1918) report the case of a dysentery-like organism which under ordinary conditions attacked glucose only but which when cultivated under diminished oxygen tension acidified galactose, maltose, levulose, mannitol, lactose and sucrose. It is very probable, as shown by recent work, that results were complicated by the buffer action of CO_2. Bron-

fenbrenner and Davis (1918) have isolated a number of organisms which when first studied fermented lactose very slowly, but whose power to attack this sugar could be progressively increased by cultivation in lactose media.

Such differences as those described may be in large measure explained as the result of a direct response to different environmental conditions, or as the normal quantitative variations exhibited by all biological reactions. In using any biochemical characteristic for differential purposes it is necessary to specify a standard set of conditions and to use modal points in the curve of distribution of quantitative values, rather than arbitrary limits, in defining groups. In connection with some of the irregularities observed it must be remembered that the complex sugars used by bacteriologists frequently prove on careful analysis to contain hexose-impurities; and that if not originally present the hexoses may be produced by excessive autoclaving. Another type of investigation indicates however that the causes of observed variations may be of a more deep seated nature. Twort (1907) studied the action of 18 colon-typhoid organisms on 49 different glucosides, and on the basis of the results obtained concluded that no significant differences existed and that all the organisms studied were "varieties or hybrids of one or more species." He then tried to produce modifications of fermentative power by cultivating various strains for long periods in media containing carbohydrates which they would not at first utilize, and found that they slowly acquired new fermentative powers when treated in this way. All the members of the paratyphoid group acquired the power to ferment sucrose, *B. typhosus* began to ferment dulcitol and lactose, the dysentery bacilli of Kruse and Flexner were able to ferment sucrose within twenty-four hours. Neisser (1906), Massini (1907), Burk (1908), Mueller (1908, 1909), and Penfold (1910, 1911a, 1911b, 1912) have described what appear to be clear-cut and definite cases of mutation in this group of organisms. The phenomena recorded are in general as follows: an organism cultivated on a solid medium containing a carbohydrate, which it normally fails to attack, produces colonies which at first appear (as indicated by the reaction of the indicator contained in the medium) as of the

usual non-fermenting type. Later however papillary projections appear on the colony, which are red if litmus be present, and plates made from these papillae yield a certain proportion of colonies which from the first exhibit vigorous fermentative power. Penfold (1910) studied Twort's lactose-fermenting strain of *B. typhosus* and found that it readily threw off non-fermenting mutants. In general however he found the fermentation of lactose to be a fairly stable character but dulcitol and rhamnose exhibited the phenomenon of mutation in a large number of strains. According to Mueller (1908, 1909), of 120 strains of *B. typhosus* all produced rhamnose-fermenting mutants. Mueller and Penfold (1912) both observed raffinose-fermenting mutants in colonies of members of the paratyphoid group. Of 34 strains of non-lactose-fermenters isolated by Penfold from feces, 21 produced fermenting mutants of some sort. The relation between the phenomenon of papilla-formation and the gradual increase in fermentative power, often observed (as by Bronfenbrenner) in liquid media, is well brought out in one of Penfold's investigations (1911a). In a dulcitol broth culture of *B. typhosus* which became acid to litmus after ten days of incubation he found, by plate cultures made from day to day, that at first fermenting strains were rare but that their number suddenly increased after the first week. Successive transfers in dulcitol broth produced a mass culture which turned litmus broth red in one day, presumably as a result of progressive selection of the dulcitol-fermenting mutant. In a later paper Penfold (1911b) shows that cultivation of certain colon and paratyphoid strains on chloracetic-acid agar produces varieties which have lost all power of producing gas in sugar media, while the fermentation of the alcohols is unaffected. Revis (1911) notes a similar phenomenon, a colon organism grown in presence of 0.05 per cent malachite green losing the power of gas production and after more prolonged treatment failing to coagulate milk and to form acid in dulcitol. Ledingham (1918) reports two highly variable strains of Flexner-Y dysentery, one of which produced mutants fermenting rhamnose and arabinose, while the other normally acidified these sugars but produced a mutant which failed to do so.

A general survey of this evidence indicates that the fermentative characters of the colon-typhoid bacteria are not only influenced by environmental conditions but also exhibit inherent powers of spontaneous mutation; yet it does not materially weaken the general value of tests of fermentative ability for systematic purposes. It is significant that spontaneous mutations are most common with dulcitol and rhamnose, carbohydrates which have been shown in comparative studies to lack the correlations with other characters which are exhibited by lactose, sucrose, xylose, and other substances; and that other modifications, such as the suppression of gas production, have generally been associated with strikingly abnormal environmental conditions. Taking the great mass of colon-typhoid strains, as they are isolated from the bodies or intestines of men and animals, and cultivated under standard conditions, fermentative characters exhibit a high degree of constancy and what is even more important a high degree of correlation with other bio-chemical and serological and pathogenic properties. It is no accident that the disease-producing organisms of the colon-typhoid series are practically without exception organisms which fail to produce gas in lactose media, but a law, which can have its basis only in the principle of phylogenetic relationship; and the same principle holds for numerous correlations which have been cited in the description of the six principal subdivisions of the colon typhoid series which have been recognized in the preceding section. Even Twort's studies, when properly analyzed, exhibit clear evidence of the general relationships of the chief groups of organisms concerned and of their progressive increase in fermentative power in proceeding from one end to the other of the series. His *B. alcaligenes* strains attacked none of the glucosides studied. With dysentery and typhoid organisms 7 per cent of his glucoside tests were positive; with the paratyphoid and Gaertner forms, 15 per cent; with colon bacilli, 46 per cent; and with *B. aerogenes* types, 65 per cent. We are dealing here with a group in which the line of evolutionary development has been marked most clearly by the progressive loss or progressive acquisition of fermentative power. Intermediate types appear at times and mutants may occur; but on the whole,

the series of forms between *B. alcaligenes* at one extreme and *B. aerogenes* at the other may be broken up into six or more main groups on the basis of fermentative ability; and the groups so constituted are true biologic entities marked by a number of independent correlated characters which can only have their origin in phylogenetic relationship.

THE CHEMISTRY OF THE FERMENTATIVE REACTIONS OF THE COLON-TYPHOID GROUP

The gaseous products of carbohydrate decomposition. The production of gas is the most obvious and striking evidence of carbohydrate decomposition and ever since Smith (1890) introduced the fermentation tube into bacteriology the appearance of visible gas in the Smith or Durham tube has been used as one of the most common criteria of fermentative activity. It is obvious that such a test is but a rough and approximate one and that no sharp line can be drawn between gas-producers and non-gas-producers, since a certain amount of gas will be necessary to saturate the liquid medium in the tube before excess gas will collect and become visible in the closed arm. Clark (1913) criticised Penfold's emphasis on the non-gas-forming mutants produced by the influence of chloracetic acid on this ground, and has pointed out that since normal *B. coli* produces a smaller amount of gas in glucose and galactose media than in dulcitol and mannitol the suppression of gas formation in the former case and not in the latter is perhaps merely due to a general weakening of the organisms concerned. The method of studying carbohydrate fermentation by examining the gas which collects over a liquid, which is exposed freely to the air at another point, is still less suited for careful studies of the composition of the gases present. Smith in his classic investigations laid stress not only upon the proportion of CO_2 to H_2 (which he reported as 1:2 for *B. coli*—Smith 1890) but also upon the quantitative determination of the total amount of gas present (which he states as 40 to 70 per cent for *B. coli*, *B. proteus* giving less and *B. cloacae* more,—Smith 1893b). It seems obvious that the amount and composition of the gas collected in the Smith tube will be governed almost as much by the solubility of the gases produced, and the rate at

which they diffuse outward from the free surface of the liquid medium, as by the inherent biochemical activities of the organisms which produce them, and measurements made in the Smith or Durham tube will always be accompanied by the loss of a large and a variable proportion of the CO_2 actually produced.

As a matter of fact, even before the introduction of the fermentation tube into bacteriology, the real composition of the gases produced in bacterial fermentation had been established by several observers who used the proper methods to secure a sample of all the gas actually produced, by cultivation in closed bulbs which were later evacuated by the air pump. As early as 1887 Hoppe-Seyler observed that calcium formate infected with river mud yielded a gas composed in equal volumes of CO_2 and H_2. Frankland and Fox (1889) were perhaps the first investigators to work with a pure culture, using a form which they isolated from sheep dung and named *B. ethaceticus*. This organism was said to be a gelatin liquefier but curiously enough gave the fermentative reactions of *B. coli* since Frankland and Lumsden (1892) and Frankland and MacGregor (1892) report an equal volume of CO_2 and H_2 produced in glucose, mannitol, and arabinose. Macfadyen, Nencki and Sieber (1891) reported that the gas produced by *B. aerogenes* was 72 per cent CO_2 and 28 per cent H_2. Frankland, Stanley and Frew (1891) estimated a ratio of CO_2 to H_2 of 10 to 8 for *B. pneumoniae*. Pakes and Jollyman (1901) also reported the 1:1 ratio in the decomposition of sodium formate by paratyphoid, colon and aerogenes types. By far the most exhaustive studies of this question were made, however, by Harden (1901, 1905) who placed the distinction between the 1:1 ratio for *B. coli* and the ratio of 2 or more parts of CO_2 to 1 of H_2 for *B. aerogenes* on a definite and solid basis.

In this country Bennett and Pammel (1896) showed that the gas formed in the Smith tube is at first only H_2 and that the ratio between H_2 and CO_2 depends mainly on the absorption of CO_2 by the liquid medium; and Longley and Baton (1907) made clear the slight value of measurements of the amount of gas and the ratio of its components in the open tube. Keyes (1909) and Keyes and Gillespie (1913) made the first exact studies in America of the actual gas production by exhaustion methods and

Rogers, Clark and Lubs extended and confirmed the work of Harden and broadened the basis of the radical distinction between the high and low ratio groups.

The acid products of carbohydrate decomposition. Studies of gas production have been given first place in this review of the subject because of the fact that gaseous products are most easily measured and have therefore attracted more general attention. A real comprehension of the processes involved demands however a knowledge of the soluble products as well.

In the classic studies of Frankland and his associates on *B. ethaceticus* it was shown that this organism when decomposing mannitol and glycerol produces chiefly ethyl alcohol and acetic acid, with smaller amounts of formic and succinic acids, the ratio of alcohol to acetic acid being 2 molecules to 1 in the case of mannitol and 3 to 1 in the case of glycerol. (Frankland and Fox, 1889). In later papers (Frankland and Lumsden, 1892, Frankland and MacGregor, 1892) it is stated that the proportion of acetic acid is higher with glucose than with the alcohols mentioned above, still higher with arabinose and highest of all with glyceric acid. When the fermentation takes place in a closed space a considerable amount of formic acid accumulates and a formula for the decomposition of mannitol is suggested as follows:

$$3\,C_6H_{14}O_6 + H_2O = C_2H_4O_2 + 5\,C_2H_6O + 5\,CH_2O_2 + CO_2$$

In the open tube it is assumed that the formic acid is promptly decomposed into H_2 and CO_2.

With *B. pneumoniae* under the conditions of ordinary cultivation Frankland, Stanley and Frew (1891) give a slightly different formula for the decomposition of mannitol

$$6\,C_6H_{14}O_6 + H_2O = 9\,C_2H_6O + 4\,C_2H_4O_2 + 10\,CO_2 + 8\,H_2$$

Frankland and his associates apparently failed to lay emphasis on the non-volatile acids, although they suggest the presence of traces of a fixed acid (probably succinic). Macfadyen, Nencki and Sieber (1891) however report ethyl alcohol and acetic and lactic acids in glucose broth cultures of colon-group organisms, the proportion of acetic acid being less in the case of *B.*

aerogenes than in cultures of *B. coli*. Grimbert (1896) gives the results tabulated below for the Friedlaender bacillus and *B. coli*.

Products of permanent activity

	GRAMS FORMED PER 100 GRAMS OF CARBOHYDRATE FERMENTED					
	B. pneumoniae				B. coli	
	Mannitol	Dulcitol	Arabinose	Xylose	Glucose	Lactose
Ethyl alcohol	11.4	29.3	0.0	6.9	Trace	6.8
Acetic acid	10.6	9.6	36.1	23.4	14.3	25.4
Laevo lactic acid	36.6	0.0	49.9	Trace	42.7	Trace
Succinic acid	0.0	21.6	0.0	19.9	Trace	29.8

Duchacek (1904) found that in media exposed to air *B. coli* produced approximately equal amounts of lactic and acetic acids, but that under an atmosphere of hydrogen the proportion of lactic acid increased to 2:1. *B. typhosus* formed a considerably higher proportion of lactic acid than *B. coli*.

Harden's work constitutes by far the most extensive investigation into this question. Harden and Walpole (1906) give the following results of the fermentative activity of *B. coli* and *B. aerogenes*.

Products of fermentative activity

	GLUCOSE		MANNITOL	
	B. coli	B. aerogenes	B. coli	B. aerogenes.
Per cent of carbohydrate fermented				
Alcohol	12.8	18.2	28.1	32.5
Acetic acid	18.8	8.6	9.5	2.1
Lactic acid	31.9	9.1	18.6	8.6
Succinic acid	5.2	4.5	8.9	2.8
Formic acid	0.0	1.7	3.0	1.6
Carbon dioxid	18.1	35.2	28.4	35.5
Cubic centimeters per gram				
CO_2	91.8	178.5	143.0	180.3
H_2	110.0	92.4	167.0	143.6
Ratio $H-CO_2$	1.19	0.52	1.18	0.79

The results for *B. coli* with glucose would correspond roughly to the following formula:

$$2C_6H_{12}O_6 + H_2O = 2C_3H_6O_3 + C_2H_4O_2 + C_2H_6O + 2CO_2 + 2H_2$$

Levulose behaves in the same way; and the general proportion of the end-products in the case of the hexoses and the alcohols is explained in the following scheme.

HEXOSE-GLUCOSE		PRODUCTS	ALCOHOL - MANNITOL		PRODUCTS
CH_2OH		C_2H_4O $+CO_2$ $+H_2$	CH_2OH		C_2H_4O $+CO_2$ $+H_2$
CHOH	CH_2OH		CHOH	CH_2OH	
CHOH	CHOH		CHOH	CHOH	
CHOH	CHOH	$2C_2H_4O_2$	CHOH	CHOH	$2C_2H_4O_2$
CHOH	CHOH		CHOH	CHOH	
CHO	CHOH				
	CHO	$C_2H_4O_2$ $+CO$	CH_2OH	CHOH	C_2H_4O $+CO_2$ $+H_2$
				CH_2OH	

For glucose this scheme would yield 2 molecules of lactic acid and 1 each of acetic acid and alcohol, for mannitol 2 each of lactic acid and alcohol; and to explain the observed results it is necessary to assume that in the latter case the lactic acid is largely destroyed by secondary reactions. Glycerol according to Harden is split directly into alcohol and formic acid.

In the case of *B. aerogenes* the proportion of lactic acid to alcohol formed is lower with glucose and very much lower with mannitol, while the acetic acid is even more reduced. It is evident that the portion of the carbohydrate molecule which furnishes the acids in the case of *B. coli* is here split in a different way and Harden and Walpole (1906) have shown that the end product in the *B. aerogenes* fermentation is chiefly 2:3-butylene glycol with some acetyl methyl-carbinol. The latter substance as shown by Harden (1906) and by Harden and Norris (1912) is the active agent in producing the Voges-Proskauer reaction. The acetyl methyl carbinol in the presence of potash and oxygen is oxidized to $CH_3.CO.CO.CH_3$ (diacetyl) and the diacetyl reacts with some constituent of the peptone medium to produce the eosin-like fluorescence characteristic of this reaction.

Harden's studies were confined for the most part to the gas-producing organisms but he points out that with forms like *B. typhosus* formic acid must be produced from the fractions of the molecule which with *B. coli* yield CO_2 and H_2. Sera (1910a, 1910b) has investigated this point and reports that typhoid and dysentery bacilli in decomposing glucose, glycerol or mannitol produce acetic and formic acids, with some propionic acid in the case of glycerol. In glucose and glycerol acetic acid is in excess, in mannitol formic acid. *B. typhosus* also forms a trace of alcohol.

An illuminating study was made by Grey (1914) of the fermentative products of Penfold's chloracetic acid mutants, in which the power of gas production had been reduced. He found that in each case the selected strains produced in glucose media more lactic acid and less alcohol, acetic acid and formic acid, and also decomposed less of the formic acid which they did produce. In mannitol however the change was limited to a decrease in decomposition of formic acid, the primary products formed being the same. Grey assumes that the glucose molecule is normally split, first into lactic acid and an Intermediate substance A, which in turn yields formic acid and an Intermediate substance B (possibly acetaldehyde). The Intermediate substance B finally yields alcohol and acetic acid. Grey believes that the decomposition of Intermediate substance B is due to a reduction, accomplished ordinarily by the excess of H_2 present in the case of mannitol but by a special reductase in the case of glucose. If the formation of this reductase were suppressed by chloracetic acid it would explain the fact that, while Intermediate substance B undergoes a normal splitting in the case of the selected strains when acting on mannitol, the process is fundamentally affected in the case of glucose. The decomposition of formic acid is of course equally affected in both instances.

It is evident that the changes which go on in the fermentation of carbohydrate media are complex and for the most part still obscure; but the broad facts appear to be established that the process leads to the production of alcohol and of acetic, lactic, formic and succinic acids (the latter in small amount); that the

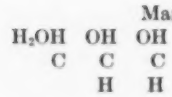
decomposition of the formic acid and perhaps of other constituents of the molecule produces a gas composed of carbon dioxid and hydrogen; that in the decomposition of mannitol alcohol is produced in much greater, and lactic acid in much smaller, amount than is the case with glucose; that in the case of *B. aerogenes* butyleneglycol, acetylmethylcarbinol and an excess of carbon dioxid are formed at the expense of the portion that with *B. coli* yields lactic and acetic acids.

Relation between the structural formula of the carbohydrates and their decomposition by colon-typhoid bacteria. The question of the relation between molecular structure and fermentability is of the greatest interest to the biologist, since data in regard to such a relationship may reasonably be expected to throw important light upon the underlying bio-chemical processes involved. The studies made upon the colon-typhoid group justify a few fundamental generalizations in regard to this point.

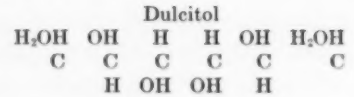
First of all it is evident that the hexoses are more easily attacked than any of the other substances ordinarily investigated. All of the members of the colon-typhoid group, with the exception of *B. alcaligenes*, produce a marked increase of acidity in these carbohydrate media. In our own studies we find that fructose, mannose, and galactose behave essentially like glucose, all the strains which acidify glucose acidifying all the other hexoses as well.

The hexahydric alcohol, mannitol, is the substance which comes next in order of availability, all of the groups studied except *B. alcaligenes* and the Shiga dysentery types acidifying this substance. Dulcitol however, which resembles mannitol so closely, is much more resistant. As a matter of fact dulcitol occupies a somewhat unique position among all the carbohydrates whose decomposition has been extensively studied. It is the only one whose fermentation seems wholly uncorrelated with that of other carbohydrate media. In all the principal groups of the paratyphoid, colon and aerogenes series we find forms which ferment this alcohol and others which fail to do so; and it has been pointed out that this particular fermentation is the one which exhibits the most general tendency toward mutative variability.

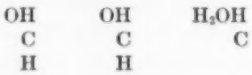
Mannitol



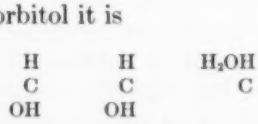
Dulcitol



Sorbitol appears to behave like mannitol, being attacked by all the paratyphoid types according to Boycott (1906), Jordan (1917), and Krumwiede Kohn, and Valentine (1918). From the formulae of these alcohols it may be noted, as suggested by Revis (1910) that the configuration of mannitol and sorbitol resembles that of the alcohol half of the hexoses. In the hexoses the grouping is



In mannitol and sorbitol it is

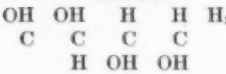


In dulcitol on the other hand there is no alcohol grouping with two adjacent carbon atoms having the hydroxyls on the same side; and in this difference may perhaps lie the difference in susceptibility to bacterial decomposition.

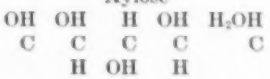
Next to these alcohols in availability for the colon-typhoid organisms comes the disaccharide maltose. We have not studied this carbohydrate ourselves but Hiss and Russell (1903), Hiss (1904), Smith (1915), and others show that the Shiga type of dysentery bacillus fails to attack maltose, while Morgan (1906, 1911) reports negative results for the peculiar organism which bears his name, as do Hadley, Elkins and Caldwell (1918) for *B. pullorum*. On the other hand Drigalski and Conradi (1902), Boycott (1906), Sacquépée and Chevrel (1905), Savage (1912), Jordan (1917), and Krumwiede, Kohn and Valentine (1918) find that the typhoid and paratyphoid organisms all attack maltose readily.

The next substances, in order of availability, appear to be xylose and arabinose with the structural formulae indicated below.

Arabinose



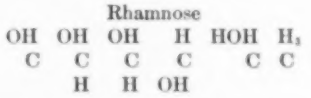
Xylose



Here we may note the very interesting fact that among the less active fermenters of the typhoid and paratyphoid series (where the fermentation of these sugars first appears) action upon xylose and arabinose seems to be inversely correlated. Thus the Flexner dysentery and paratyphoid A types attack arabinose but not xylose, the typhoid and hog-cholera strains xylose but not arabinose. It would appear that the power to ferment the two different molecular groupings represented by these sugars appears or disappears independently and they are presumably attacked in a quite different way.

Rhamnose is the next carbohydrate to be attacked by the colon-typhoid bacteria.[2] This substance, which is a methylated pentose, is utilized by all the paratyphoid, colon and aerogenes series; and it may be assumed that the presence of the methyl group is the circumstance which prevents the Flexner dysentery and typhoid organisms from decomposing it.

Rhamnose



From the five and six carbon carbohydrates to the disaccharide lactose there is a distinct and well marked step; and we may reasonably assume that the broad distinction between the pathogenic dysentery-typhoid-paratyphoid series on the one hand and the non-pathogenic colon-aerogenes series on the other is correlated with the absence or presence of an enzyme capable of breaking up this disaccharide into glucose and galactose. The

[2] In this discussion we have for convenience considered the carbohydrates in their order of availability, passing from those most easily to those least commonly fermented. It is not necessary however to assume that evolution has proceeded along the line of a progressive acquisition of fermentative properties. Since the organisms of low fermentative power are the pathogenic (and presumably more recent) forms it would be more reasonable to assume that development has been in the direction of a loss rather than a gain in fermentative power, in which case *B. aerogenes* might be the primitive ancestral type of the whole group.

difference between maltose and lactose in availability is a striking and significant one. It has been noted that all the members of the typhoid and paratyphoid series produce acid from maltose; but the power to decompose lactose appears only among the gas-producing forms of the colon and aerogenes groups.

It seems clear that the processes involved in the decomposition of the two disaccharides, maltose and lactose, must be fundamentally dissimilar; and it is of interest to note that while the difference in availablity between mannitol and sorbitol and between the three pentoses are associated with differences in reactive organic groups, the even more fundamental difference between the availability of maltose and lactose can be explained only by a stereoisomeric difference in the molecules concerned. As in the case of animal enzymes the presence of maltase and lactase seems to vary quite independently.

Next to lactose in order of availability come sucrose and raffinose; and here we find another definite relation between fermentability and molecular structure. The correlation between the fermentability of these two sugars has been shown by Winslow and Walker (1907), Burk (1907), Howe (1912), Rogers, Clark and Davis (1914a), Kligler (1914), Levine (1916) Murray (1916), and Rogers, Clark and Lubs (1918) to be an almost perfect one. These two sugars, although one is a disaccharide and the other a trisaccharide, are alike in the absence of the aldehyde group; and this fact may be assumed to account for the similarity in their behavior.

The fermentation of the glucoside salicin is generally, but not always, correlated with that of lactose; being fermented by most, but not all, of the colon group and by all the *B. aerogenes* strains; while the cyclohexanhexol inosite appears to be fermented only by Paratyphoid B and certain *B. aerogenes* strains.

PROGRESSIVE CHANGES OF REACTION IN CARBOHYDRATE MEDIA AND THEIR SIGNIFICANCE

The estimation of the individual products of carbohydrate decomposition is of course a difficult and time consuming process; and even the determination of the gases evolved must be

carried out by exhaustion from a closed vessel if it is to have any serious value. The acidity produced in a given culture medium is on the other hand a characteristic which can be easily and accurately measured. To this point we have therefore devoted a considerable share of our attention.

When the work on the colon-typhoid group was begun we made our first studies of acid production by the old titration method, cultivating each strain in broth (containing 1 per cent Digestive Ferments Company peptone, 0.5 per cent K_2HPO_4, and 1 per cent of carbohydrate) at 30°C. After two days and seven days of incubation, tubes were withdrawn, held in steam or boiling water for ten minutes to drive off CO_2, cooled and titrated against N/20 NaOH with phenolphthalein as indicator. The general results of the observations made by this method on a series of colon-aerogenes strains have been already reported by one of us (Kligler, 1914). They indicated that in any carbohydrate medium there is a fairly sharp distinction between fermenting and non-fermenting forms, the line between the two groups being at about 1.5 per cent normal acid, (as ordinarily determined), the mode for the non-fermenters being near the neutral point and that for the fermenters varying between 2.5 and 3.5 per cent acid for the various carbohydrates.

In view of the rapidly accumulating evidence which indicated that the measurement of acidity by the titration method is inaccurate and often misleading, we determined the true acidity in the various fermentable media for our complete series by the use of the Clark and Lubs indicators. The media first used for this purpose contained 0.5 per cent Digestive Ferments Company peptone 0.5 K_2HPO_4 and 0.5 per cent of the fermentable substance to be studied, as recommended by Clark and Lubs. We found however that a somewhat sharper differentiation could be obtained by increasing the amount of sugar, and in our regular routine studies the medium was made up with 0.5 per cent peptone, 0.5 per cent K_2HPO_4 and 1 per cent of the fermentable substance. The cultures were incubated at 30°C. and the acidity was determined after two, four and five days by the use of methyl red, dibromthymolsulphonphthalein and phenol sul-

phonphthalein, according to the methods suggested by Clark and Lubs (1917).

In general four distinct types of reaction were observed when the cultures were examined in this way, reactions which we have described as types I, II, III, and IV (Winslow, Kligler and Rothberg, 1917). In type I which is observed when the carbohydrate present is not attacked at all, the reaction remains throughout in the neighborhood of P_H 7.0. Type II is the reaction characteristic of slow fermentation, the P_H value after two days being still at 6 or over, and only later reaching an acidity below 5.5. This type of reaction appears with substances which are decomposed with some difficulty, such as dulcitol, rhamnose, and salicin, and the principal results of this kind which we obtained are tabulated below.

Type II reactions

GROUP OF ORGANISMS	FERMENTABLE SUBSTANCE	AVERAGE P_H VALUES AFTER		
		2 days	4 days	5 days
Flexner dysentery	Mannitol	6.5	5.5	5.7
Paratyphoid A	Rhamnose	6.4	5.0	5.1
	Arabinose	7.0	5.0	5.1
B. suipestifer	Rhamnose	7.0	6.4	5.8
Paratyphoid B	Rhamnose	6.7	5.0	5.1
	Dulcitol	6.7	5.3	5.2
B. communior	Dulcitol	7.0	5.6	5.5
	Salicin	5.9	5.1	4.8
B. communis	Sucrose	6.1	5.2	5.2
	Salicin	6.4	5.4	5.3
	Dulcitol	7.1	5.6	5.5

A type III reaction is the normal reaction for the colon-typhoid organisms in any carbohydrate which is readily fermented. In this case the acidity rises rapidly, usually within twenty-four hours and always within forty-eight hours, to a P_H value of 5.3 or less and remains practically constant at about that point.

We have observed this rapid rise and subsequent maintenance of high acidity with all the groups of organisms studied (except *B. aerogenes*) and in the case of all the substances fermented except those cited above as giving a somewhat delayed reaction. With these exceptions the P_H value after two days of incubation varied for all the groups studied and for all the substances fermented between the limits of 5.0 and 5.6, while the final values after five or six days varied between 4.8 and 5.3. This final reaction averaging about 5.0 is so constant that it may be fairly assumed to represent the limiting concentration of acid for the colon-typhoid organisms in the particular medium used for our studies. Clark (1915) has pointed out the same constancy in final reaction for *B. coli* in 30 different media, his range of variation (even with a series of variously buffered media) being only from P_H 4.3 to P_H 5.3. Michaelis and Marcora (1912) reported 4.8 as the limiting P_H value for *B. coli* in glucose peptone broth. Our results make it possible to extend these conclusions and to state that not only for *B. coli* but for the whole colon-typhoid group the final acidity in sugar broths is practically the same,—about P_H 5.0—in the medium studied by us.

In a longer period of incubation than that used for our routine determinations (five days) there appears to be a slight reversion toward alkalinity even in the case of the type III reaction, as indicated by the results tabulated below for a series of 9 typical typhoid strains; and this slight reversion may probably be attributed to the formation of alkaline products of protein decomposition.

Average acidity (PH) *produced after various periods by nine typhoid strains*

	1 DAY	2 DAYS	4 DAYS	6 DAYS	8 DAYS	11 DAYS	13 DAYS
Glucose	5.3	4.9	5.0	5.2	5.3	5.3	5.4
Mannitol	5.5	5.1	5.4	5.6	5.8	5.9	5.8

It will be noted that the P_H values are slightly higher throughout in the mannitol as compared with the glucose broth. Our results indicate that there exist minor differences of this kind, the fermentation of carbohydrates which are less readily utilized being a trifle slower and producing a little lower acidity than the

decomposition of glucose. The line between a type II and a type III reaction is not therefore an absolutely sharp one.

The type IV reaction, in which the acidity rarely goes below a P_H value of 5.5 and quickly reverts to a value over P_H 6 is characteristic of the *B. aerogenes* group and appears to be related to a fundamentally different action upon the fermentable substances. The differences between the type III and type IV reactions during the first forty-eight hours are indicated below for a series of *B. coli* and *B. aerogenes* strains.

Average acidity (PH) *produced by a series of strains of B. coli and B. aerogenes in glucose broth*

	2 HOURS	4 HOURS	8 HOURS	24 HOURS	48 HOURS
B. coli	7.3	7.2	6.0	4.9	4.7
B. aerogenes	7.2	6.8	5.8	5.7	6.2

The reactions cited below for *B. aerogenes* during a longer period of incubation in various carbohydrate media are for a selected series of 10 typical strains and may be compared with corresponding figures quoted above for *B. typhosus* in glucose and mannitol.

Average acidity (PH) *produced by a series of B. aerogenes strains*

	1 DAY	2 DAYS	4 DAYS	6 DAYS	8 DAYS	11 DAYS	13 DAYS
Glucose	5.3	5.3	5.8	6.2	6.3	6.6	6.3
Lactose	5.6	5.6	5.9	5.9	6.1	6.8	7.0
Sucrose	5.4	5.5	6.0	6.4	6.5	7.1	6.8
Rhamnose	5.5	5.4	5.7	5.1	5.9	6.3	6.3

In the type IV reaction, marked differences in rate of fermentation appear between individual strains and a high acidity (in some cases as high as P_H 5.1) may be attained for brief periods. The reversion is very rapid however and P_H values lower than 5.5 are rarely observed.

The phenomenon of reversion from a preliminary acid reaction to a more alkaline one was explained by the earlier workers on this subject as due to exhaustion of the carbohydrate, followed by the utilization of nitrogenous foodstuffs with the release of alkaline products of protein decomposition.

Kendall, Day and Walker (1913) made a somewhat extensive study of this point, determining the reaction of various members of the colon-typhoid group in plain broth and glucose broth by the use of neutral red as an indicator, and simultaneously estimating the rate of protein decomposition by the increase in ammonia. Their results indicated a definite sparing action of the sugar. For example *B. dysenteriae* in plain broth produced 2.1 to 4.2 mgm. of NH_3 per 100 cc. broth with no material change in reaction, while in 1 per cent glucose broth the reaction reached an acidity of 2 to 2.8 per cent Normal to neutral red, while no ammonia was formed. *B. typhosus*, the paratyphoids, the Morgan bacillus, and *B. coli* all behaved in essentially the same way; while in the case of *B. cloacae* the carbohydrate was apparently destroyed so quickly that it made little difference in the protein metabolism whether glucose was present or not. In the case of *B. alcaligenes* there was no difference in reaction between the plain broth and the glucose broth, but growth was more luxuriant and more ammonia was produced in the latter case, suggesting some sort of utilization either of the glucose itself or of impurities contained therein. The range of values recorded for certain groups are of such interest as to warrant reproduction.

ORGANISM	BROTH	REACTION TO NEUTRAL RED	INCREASE IN NH_3
B. dysenteriae	Plain	−0.3 to +0.2	2.1 to 4.2
	Glucose	2.0 to 2.8	None
B. typhosus	Plain	+0.1 to −0.9	4.2 to 10.5
	Glucose	2.9 to 4.9	None
Paratyphoids	Plain	−1.5 to +0.3	4.2 to 16.1
	Glucose	2.3 to 4.8	0.0 to 2.8
Morgan bacillus	Plain	−0.5 to −2.1	20.3 to 42.0
	Glucose	2.9 to 4.6	4.2 to 8.4
B. coli	Plain	−0.4 to 1.9	11.2 to 36.4
	Glucose	3.9 to 6.1	−1.4 to 2.1
B. cloacae	Plain	−0.9 to −1.4	20.3 to 21.7
	Glucose	−0.6 to +1.0	16.8 to 19.0

These results do indeed suggest that the difference in reaction in a carbohydrate medium may be partly due to the relative rate at which protein and carbohydrate, respectively, are being attacked. That in the case of certain organisms there is something much more fundamental involved is indicated by the peculiar behavior of *B. cloacae* (a form which belongs to the *B. aerogenes* group); and the same deep-seated difference is indicated by the progressive change of reaction in glucose broth recorded by Kendall, Day and Walker for the various groups of organisms studied. We have calculated below the average values for certain of these groups and in so doing have separated one of the groups presented by the authors under the name of *B. mucosus-capsulatus* into two subgroups since 9 strains called by Kendall, Day and Walker *B. lactis-aerogenes* are clearly different in their behavior from the rest of the *B. mucosus-capsulatus* groups.

Average acidity (to neutral red) in glucose broth cultures of various colon-typhoid organisms after various intervals

DAY	B. ALCALIGENES	DYSENTERY	TYPHOID	PARATYPHOID	MORGAN	B. COLI	B. LACTIS AEROGENES	B. MUCOSUS CAPSULATUS	B. CLOACAE
1	− 0.4	2.1	3.0	3.0	2.8	3.8	3.7	2.2	2.0
3	− 0.8	2.7	3.3	3.8	3.8	4.4	4.2	1.6	1.6
6	− 1.0	2.7	3.5	3.8	3.7	4.8	4.8	1.3	0.9
9	− 1.1	2.7	3.6	3.7	3.9	5.0	4.6	1.0	− 0.1

The results, as tabulated above, indicate a progressive increase in acidity (a type III reaction) for dysentery, paratyphoid, Morgan, typhoid and colon bacilli, although it is interesting to note that Kendall, Day and Walker's figures indicate a distinct difference in the final end-point, the dysentery bacilli producing a lower, and the colon bacilli a higher, acidity than the typhoid, paratyphoid and Morgan groups. The forms called by Kendall, Day and Walker *B. lactis-aerogenes* appear by their fermentative activity to be of the *B. coli* type but those designated as *B. mucosus-capsulatus*, as well as the *B. cloacae* forms, show the characteristic type IV reaction of *B. aerogenes*.

Ayers and Rupp (1918) have recently brought forward direct and convincing evidence that the reversion of reaction exhibited in *B. aerogenes* cultures is due rather to the secondary decomposition of organic acids with the formation of basic carbonates than to neutralization by basic products of protein decomposition. They show that the reversion takes place even in a synthetic medium containing sodium ammonium phosphate as a source of nitrogen (in which case ammoniacal products could not possibly be sufficient to account for the phenomenon); and that it is accompanied by a rapid destruction of formic, acetic and other acids. Even with *B. coli* they noted as might be expected that acid formation does not run parallel with the destruction of glucose, formic acid remaining constant or being slightly reduced during the later stages of fermentation. The distinction between *B. coli* and *B. aerogenes* may therefore be considered as lying chiefly in the difference in rate between the preliminary decomposition of the sugars into acids and the secondary decomposition of the acids themselves; yet it is evidently a distinction that is clearly marked and of important systematic significance.

The slower and much less marked reversion which is observed if organisms giving the type III reaction are cultivated for a prolonged period may perhaps be due to a different process, an exhaustion of the fermentable carbohydrate and a subsequent decomposition of nitrogenous foodstuffs with the formation of alkaline substances. The same explanation may probably account for the reversion shown in milk cultures by the members of the paratyphoid group, the transient acidity produced being due to the fermentation of the small amount of glucose present.

It must of course be remembered that the course of such reactions as those described will vary within wide limits with variations in the composition of the medium employed. Clark and Lubs (1915) studied the effect of varying the concentration of carbohydrate and showed that with 0.5 per cent peptone and 0.5 per cent K_2HPO_4 and 0.1 per cent glucose *B. coli* shows a marked reversion, the curve of acidity by days resembling closely that given by *B. aerogenes* in the presence of 0.5 per cent glucose.

It has been shown by one of us (Kligler 1916) that the net result of the action of bacteria in a given medium not only depends on the relative amounts of peptone and glucose present but is influenced by the amount of phosphate as well.

CHARACTERISTICS OF COLON-TYPHOID BACTERIA OF GROUP I (FERMENTING NO CARBOHYDRATES MORE COMPLEX THAN THE HEXOSES)

The first well marked group of colon-typhoid organisms, as pointed out above, includes the forms of exceedingly low fermentative power which are capable of attacking only the simple hexoses if they ferment any carbohydrates at all, but are unable to produce acid in mannitol media.

The collection of cultures studied by us included 14 different strains belonging to this general group, which could be subdivided further by their action upon the hexoses and the formation of indol.

Five of the strains formed no acid in any carbohydrate media, the P_H value of glucose broth remaining over 7.0 and milk cultures turning gradually to a deeper and deeper blue.[3] One strain (no. 439) showed a decolorization in milk after four weeks incubation, the color changing from deep blue to a pale chalky tint. These cultures were sent in to the Museum collection with the following names: *B. pneumoniae, B. dysenteriae, B. alcaligenes, B. ozenae, B. bronchisepticus;* but all of them have lost the power of attacking carbohydrates if they ever possessed it. All are indol negative[4] and all fail to liquefy gelatin.[5]

[3] Observations of the behavior of our cultures in milk were made under the following conditions. Certified milk was steamed for forty-five minutes in the Arnold sterilizer and left over night in the ice box for the cream to rise. The milk was then siphoned off, tinted with Kahlbaum's azolitmin, tubed and sterilized in the Arnold for twenty minutes on three successive days. After inoculation the cultures were incubated at 37°C. and observed after twenty-four and forty-eight hours and one, two, four and six weeks.

[4] Indol production was studied by the following method which Marshall (1907) and others have shown to be an accurate and satisfactory one. A medium containing 0.3 gram tryptophane and 5 grams K_2HPO_4 in 1000 cc. of water was inoculated from a fresh twenty-four hour culture and incubated at 30° for forty-

It is of some interest to note that the names borne by three of these five strains would indicate that they did originally possess fermentative powers. Strain 27 of our collection was obtained from The Rockefeller Institute, as a Kral culture of *B. pneumoniae*; Strain 543 was isolated at the University of Pennsylvania, from a case of atrophic rhinitis identified as *B. ozenae* and successfully used as a vaccine; strain 177 was described as *B. dysenteriae* in the Journal of Experimental Medicine, **6**, 181.

We have made no tests of motility on our strains, but Kendall, Day and Walker (1913) and Stewart (1917) describe *B. alcaligenes* as motile. Kühnemann (1911) claims that it has polar flagella, which if correct would remove it from the colon-typhoid group entirely. Petruschky's original description includes the statement that the flagella are peritrichic, and stresses the brownish growth upon potato. Berghaus (1905) who studied *B. alcaligenes* with some care bases the characterization of this species on the strongly alkaline reaction in milk, a brown coloration on potato, specific agglutination reactions, and the fact that it is an obligate aerobe. The species may be described as follows, and strain 439 of the American Museum collection (isolated from feces at the University of Pennsylvania) may be taken as a type.

B. alcaligenes Petruschky. Gram-negative non-spore-forming motile rods, producing thin translucent irregular colonies on

eight hours. One cubic centimeter of a 2 per cent alcoholic solution of p-dimethylamidobenzaldehyd was added drop by drop so as to mix with the medium, followed by a few drops of concentrated HCl, a reddish-purple color indicating the presence of indol. A comparison of the results obtained over a year before with the same cultures in a pepton medium showed that the results with the two procedures were identical.

[5] Gelatin liquefaction was observed in small test tubes 1 cm. in diameter and 10 cm. long. Five cubic centimeters of standard gelatin made with Liebig's beef extract was placed in a tube and sterilized in the autoclave for five minutes under 20 pounds pressure. The tubes were inoculated by spreading a loopful of a twenty-four hour broth culture over the surface of the medium and they were incubated for twenty days at 20°C. in an atmosphere saturated with moisture. At the end of this time the depth of liquefied gelatin was estimated in centimeters.

gelatin and a brownish growth on potato. Reaction in carbohydrate media alkaline. Indol not produced. Gelatin not liquefied.

A second subdivision of the non-mannitol fermenting organisms, characterized by the decomposition of the hexoses with the production of acid but no gas, was represented in our collection by but two strains, the dysentery bacilli of Shiga and Kruse, respectively. They gave a type III reaction in glucose, mannose, fructose and galactose as indicated by the figures cited below.

Reaction (PH) *produced by non-mannite-fermenting dysentery bacilli in glucose broth*

	2 DAYS	4 DAYS	5 DAYS
Kruse	4.9	4.9	4.9
Shiga	5.0	5.1	4.9

No other carbohydrates were fermented; and in milk both strains produced a very faint initial reddening followed by a return to the original color of the medium. Indol was not formed, gelatin was not liquefied, and lead acetate media were not turned brown. Shiga originally described his dysentery organism as slowly motile but Kruse (1900), Vedder and Duval (1902) and all later observers agree that it is non-motile. All investigators who have studied the agglutination reactions of dysentery bacilli (among whom may be mentioned Pai and Krishnan, 1916 and Andrewes, 1918) report that the agglutinations of the Shiga type (including the Kruse and New Haven strains of non-mannite-fermenters) are sharply specific. Furthermore, as shown by Kruse, Flexner and Sweet (1906) and others the Shiga bacillus possesses the power, unique in the colon-typhoid group, of producing a soluble toxin. Nicolle, Debains and Loiseau (1916) note that this type produces in culture a characteristic odor of chestnut flowers. The Shiga type of dysentery appears to be an unusually definite and distinct one. Martin and Williams (1917) for example isolated 47 strains at

Cairo, all of which were true to type. While it is of course certain that both the mannit fermenting and non-mannit-fermenting types of dysentery bacilli are causally connected with the disease whose English name they bear, it is most desirable that they should be given definite Latin names in accord with scientific usage—particularly if, as we believe, they represent quite sharply differentiated bacterial types. It seems to us that the Flexner organism for instance is biologically more nearly related to *B. typhosus* than to the Shiga bacillus. Chester (1901) has given the Shiga type the name *B. shigae* and it may be characterized as follows.

B. shigae Chester. Gram-negative, non-spore-forming non-motile rods, producing thin translucent irregular colonies on gelatin. Form acid rapidly in media containing the hexoses, but not in other carbohydrates. Milk turned slightly acid and then neutral or slightly alkaline. Indol not produced. Gelatin not liquefied. Lead acetate media not browned. Produces a soluble toxin and exhibits characteristic agglutinative reactions. Found in human stools. The causative organism in one form of dysentery. Strain 197 of the American Museum collection is a type of this species. It was obtained by us from The Rockefeller Institute, labeled "Shiga, Japan, 293."

CHARACTERISTICS OF COLON-TYPHOID BACTERIA OF GROUP II (FERMENTING MANNITOL BUT NOT RHAMNOSE)

The second general group recognized above includes the forms which ferment mannitol and either xylose or arabinose but rarely both; and which produce acid but not gas in the sugars which are attacked. The arabinose-positive forms correspond to the Flexner group of dysentery bacilli and the arabinose-negative forms to *B. typhosus*.

Of the arabinose-fermenters we had only two strains in our series, both obtained from The Rockefeller Institute, strain 110 a Flexner-Harris strain, isolated by Flexner at Manilla in 1900 and strain 196, a strain also isolated in the Philippines by Strong. Both were alike in forming acid in glucose, mannose, fructose,

galactose, mannitol and arabinose, but not in the other carbohydrates studied. The acid production was however rather of our type II than our type III order, being slightly more sluggish than in the case of most of the organisms studied, as indicated below.

Acidity (Pʜ) *produced by Flexner dysentery bacilli in glucose broth*

	2 DAYS	4 DAYS	5 DAYS
Strain 110	6.0	5.4	5.5
Strain 196	5.6	5.4	5.6

Milk cultures were practically unchanged, although strain 110 showed a very slight reddening after two days, with reversion to the original neutral tint. Indol was produced and gelatin was not liquefied. Lead acetate media were not turned brown.

While the Shiga dysentery organisms constitute a clear and well-defined type the mannitol-fermenting strains are much more variable. Lentz reported that the Flexner strains fermented maltose and dextrin, while his Strong strains (all mannitol-fermenters) failed to do so. Hiss and Russell (1903) described their Y strain as failing to attack maltose; while the Flexner strain they studied acidified both maltose and sucrose. Park, Collins and Goodwin (1904) reported the same reactions for the Flexner type, while their Seal Harbor strain failed to attack either maltose or sucrose. Hiss (1904) recognized three subgroups of mannitol-fermenting dysentery bacilli as follows:

TYPES	MALTOSE	SUCROSE	DEXTRIN
Y (Hiss), Seal Harbor (Park), etc.	−	−	−
Flexner, Strong	−	+	−
Flexner, Harris, Wolstein	+	+	+

On prolonged cultivation however the Y strains did attack maltose and sucrose (and a non-mannitol-fermenting Kruse strain attacked maltose!). Gay (1904) gave a similar classification. Morgan (1911) examined a series of about 50 dysentery-

like organisms, half from England and half from abroad and reported a considerable proportion of positive results (acid-production) in media containing sorbitol, arabinose and raffinose, in addition to the carbohydrates included in the table above. He concludes that

> In the mannite-fermenting dysentery group (excluding the "Strong" strains) must be incorporated a large and probably ever-increasing number of strains reacting with striking uniformity to one test (i.e. agglutination with "Y" or Flexner serum) but differing from one another markedly when their fermentation properties and receptor mechanisms are minutely investigated.

Smith (1913) gives an elaborate classification of this group based on reactions in dulcitol, sucrose, sorbitol, dextrin and maltose. He calls the Y type dextrin positive, and in a later paper (Smith, 1915) gives the following reactions for the three common types.

	DULCITOL	MALTOSE	SORBITOL	DEXTRIN	ARABINOSE	RAFFINOSE
Y (Hiss)	−	−	−	+	+	+
Strong	+	+	+	−	+	+
Flexner-Harris	−	+	−	+	+	+

It will be noted that these results differ in some respects from those cited above from Hiss and Gay, and it is probable that there were errors in the work of the earlier observers. Hort (1915) gives the same characteristics as those tabulated by Smith.

Recent observers have emphasized particularly the variability of the members of the mannitol-fermenting dysentery group and the difficulty of drawing sharp lines of distinction between them. Thus Pai and Krishnan (1916) report that Flexner and Y types cannot be clearly distinguished by their agglutination reactions. Martin and Williams (1917) studied 76 mannitol-fermenting dysentery bacilli which were constantly positive (acid-producing) in glucose, galactose and mannitol and constantly negative in lactose, dulcitol, inulin and adonitol, but which varied widely in their action on maltose, sucrose, dextrin,

raffinose, arabinose, rhamnose, sorbitol, glycerol and indol,—the same strain giving variable results at different times. Andrews (1918) and Thøtta (1919) have recently described types of dysentery-like organisms which indicate a confusing series of varieties within this group. Strain 110 of the American Museum collection, as originally a Flexner strain, should ferment maltose but as a matter of fact does not. Strain 196 as a Strong strain should ferment both maltose and sucrose but does not, giving us further evidence of the instability of fermentative powers of these organisms.

On the whole we are inclined to think that it is wiser not to burden the literature of this highly unstable group with a multiplicity of specific names based on fermentative irregularities, and believe that it would probably be best to include all the mannitol fermenters under the species *B. dysenteriae* with the principal sub-types as varieties. It may be noted that the agglutinative characters and pathogenic properties of these organisms are much more stable and appear to warrant the recognition of the group as a distinct entity.

The species and varieties may be defined as follows:

B. dysenteriae (Flexner). Gram negative, non-spore-forming rods. Non-motile. Producing characteristic translucent irregular colonies. Gelatin not liquefied. Indol generally produced. Acid production in media containing the hexoses and mannitol, and usually in arabinose. Lead acetate media not browned. Found in stools, causal agents of one form of dysentery.

Variety Hiss-Y. Dextrin fermented, maltose not fermented, sucrose not fermented.

Variety Flexner. Dextrin and maltose both fermented. Sucrose not fermented.

Variety Strong. Maltose and sucrose fermented but not dextrin.

The biological relationships of *B. dysenteriae* (Flexner) are very hard to surmise. It is related to *B. shigae* by lack of motility and by the nature of the disease which it produces; it is related to *B. typhosus* by the fermentation of maltose and mannitol; it is allied to both these organisms by failure to produce gas. On

the other hand the fact that it forms indol and sometimes attacks such complex carbohydrates as sucrose and raffinose would suggest a transition to *B. coli* and the lactose fermenters. Andrewes' (1918) three species of non-pathogenic *B. dysenteriae*-like bacilli may be connecting links along such a line.

Turning now to the organisms which ferment xylose but not arabinose, (*B. typhosus*) we find a much more compact and homogeneous group.

The typhoid bacillus, as pointed out in an earlier section, is characterized by active motility, failure to produce indol, and failure to ferment the more complex carbohydrates such as arabinose and rhamnose. Of our series of cultures, 24 belonged to this group. The fact that all but one of these strains came in to the American Museum collection under the name *B. typhosus* (the single exception being called *B. paratyphosus*) is good evidence of the fixity of this type. All of our 24 strains were alike in producing a rapid and complete acid reaction (type III) in the hexoses, and in mannitol, and xylose (in xylose the reaction reverted later slightly to about P_H 5.7), and in failing to attack lactose, sucrose, arabinose, rhamnose, dulcitol, or salicin. In litmus milk the medium was slightly reddened after two days but after two weeks turned neutral or slightly alkaline again. Indol was never formed, nor was gelatin liquefied. Lead acetate media were turned brown.

Sorbitol, which we did not study, is reported as acidified (Morgan, 1906, Smith, 1913, Robinson, 1915). So are maltose and dextrin (Smith, 1915, Harding and Ostenberg, 1912, Robinson, 1915). Smith (1915) states that raffinose is also attacked. This latter reaction has not been reported by other observers and seems improbable from the negative results in sucrose.

The typhoid bacillus is evidently a clearly marked and usually stable organism which may be defined as follows.

B. typhosus (Zopf). Gram-negative non-spore-forming rod. Actively motile. Forms translucent irregular colonies on gelatin media and faint nearly colorless growth on potato. Produces strong and prompt acid but no gas in media containing the hexoses maltose, mannitol, sorbitol, xylose and dextrin. Does

not attack arabinose, rhamnose or lactose. Produces a slight initial reddening of litmus milk, which after two weeks reverts to a neutral or slightly alkaline reaction. Fails to form indol or liquefy gelatin. Will not grow in asparagin-mannitol medium (Capaldi-Proskauer No. I). Does not reduce neutral red. Does cause browning of lead acetate media. Has low tolerance for acids but rather high tolerance for brilliant green dyes and alkaloids. Characteristic serum agglutination reactions. Found in human stools and urine as actual or potential cause of typhoid fever..

Our type of *B. typhosus* is strain 608, the Rawlings strain, obtained by Col. F. F. Russell from Colonel Leishman in 1908 and used in preparing the standard army vaccine.

There were two peculiar strains in our collection, both of which were sent in labeled *B. pyogenes-foetidus*. They were alike in giving the general fermentative reactions of *B. typhosus* but liquefied gelatin and casein rapidly.

CHARACTERISTICS OF COLON-TYPHOID BACILLI OF GROUP III (FERMENTING MANNITOL, ARABINOSE AND RHAMNOSE, GENERALLY FORMING GAS)

We may turn next to group III (see page 439) which includes the paratyphoid A types, fermenting the hexoses mannitol, maltose, rhamnose and arabinose (but not xylose) with the formation of gas, and producing like *B. typhosus* only a very slow reversion to an alkaline reaction in milk. Nine of the strains studied by us fell in this group. Six of them were sent in to the Museum collection labeled as Paratyphoid or specifically as Paratyphoid A; two as *B. pullorum*; and one as *B. alcaligenes*.

All fermented the hexoses, mannitol, rhamnose and arabinose. Two strains (nos. 294 and 322) attacked dulcitol. (Dulcitol is fermented by paratyphoid A according to Boycott, 1906, Morgan, 1906, Springer 1911, Smith 1915, and Jordan 1917). None of our strains produced acid in xylose, lactose, sucrose, salicin or inosite. Of the carobohydrates not studied by us, maltose is fermented by paratyphoid A according to Boycott 1906, Sac-

quépée and Chevrel 1906, Springer 1911, Smith 1915, Jordan 1917, and Krumwiede, Kohn and Valentine 1918; while dextrin is not attacked according to Jordan 1917, Weiss and Rice, 1917, Hulton-Frankel 1918 and Krumwiede, Kohn and Valentine 1918; and raffinose, inulin, erythritol and adonitol are not attacked according to Jordan 1917. Litmus milk was first turned slightly red but in the case of most of our strains by two weeks had reverted to a neutral or slightly alkaline reaction, very much as in the case of *B. typhosus*. Jordan (1917) states that his strains remained acid for two weeks, while Krumwiede, Pratt and Kohn (1916 b) report limits varying from five days to six weeks. Neutral red is said not to be reduced by Bainbridge 1919, but Sacquépée and Chevrel 1906, Hollande and Beauverie 1915, Nicolle, Raphael and Debains 1917 all report that this dye is decolorized. Lead acetate media were not browned by our cultures (as reported by Sacquépée and Chevrel 1906 and Hollande and Beauverie 1915). Gelatin was not liquefied and indol was not produced by any strain. Morgan (1906) stated that paratyphoid A produced indol but as shown by Marshall (1907) and Zipfel (1912) his methods of determining indol were unreliable. Among the minor characteristics of the paratyphoid A group may be mentioned the formation of agar colonies of a type intermediate between those characteristic of *B. typhosus* and *B. coli* respectively (Jordan 1917).

The designation of the paratyphoid bacilli as "A" and "B" has become firmly established in medical literature; but this terminology is quite clearly inadmissible from a systematic biological standpoint. The two forms are not varieties of one species but quite distinct forms, in spite of the fact that they produce a clinically similar disease. The A type is more nearly related to *B. pullorum* than to the B type; and the B type more nearly related to *B. enteritidis* and *B. suipestifer*, than to the A type of paratyphoid. It seems to us clear that definite specific names in a proper Latin form should be given to these organisms; and the name *B. paratyphosus* may properly be adopted for the A type to be defined as follows.

B. paratyphosus. Gram negative, non-spore-forming motile rods. Colonies on gelatin somewhat intermediate between the thin translucent irregular colonies of *B. typhosus* and the convex regular colonies of *B. coli* Produces acid and gas in media containing the hexoses, mannitol, rhamnose, arabinose, maltose, sorbitol and sometimes dulcitol; but not in xylose, lactose, sucrose, salicin, inosite, dextrin, raffinose, inulin, or adonitol. Milk first turned slightly red and later (usually only after two weeks) neutral or slightly blue. Neutral red reduced, lead acetate media not browned. Gelatin not liquefied. Indol not produced. Characteristic serum agglutination reactions. Found in human stools and urine as actual or potential cause of one form of paratyphoid fever.

Culture 16 in our collection has been taken as a type of *B. paratyphosus.* It was obtained from the Rockefeller Institute in 1911 labelled Schottmüller A.

Clearly allied to *B. paratyphosus* is *B. pullorum,* described by Rettger in 1900 as the causative agent in bacillary white diarrhea of chicks. This organism, as shown by Rettger and Koser (1917), Hadley, Caldwell, Elkins and Lambert (1917) and Hadley, Elkins and Caldwell (1918) has all of the ordinary cultural and fermentative reactions of the A paratyphoids except that it is non-motile and fails to attack maltose or dulcitol, and that its agglutinative relations are closer with *B. typhosus* than with any of the paratyphoids. It may be defined as follows.

B. pullorum Rettger. Gram negative non-spore-forming non-motile rods. Colonies on gelatin somewhat intermediate between the thin translucent irregular colonies of *B. typhosus* and the convex regular colonies of *B. coli.* Produces acid and gas in media containing the hexoses, mannitol, rhamnose, arabinose and sorbitol; but not in maltose, dulcitol, xylose, lactose, sucrose, salicin, inosite, dextrin, raffinose, inulin or adonitol. Milk first turned slightly acid, later, but only slowly, neutral or slightly alkaline. Lead acetate not reduced. Gelatin not liquefied. Indol not produced. Exhibits group agglutination with *B. typhosus.* Causative agent of bacillary white diarrhea

in young chicks and found (without definite pathological symptoms) in ovaries of adult fowls.

Our strain 277 is a type of this species. It was isolated by Jones at Ithaca and sent to us by Parke Davis and Company in 1911 with the number 0233.

A special variety of this species has been described by Hadley (Hadley, Caldwell, Elkins and Lambert, 1917) as causing disease in adult fowls, differentiated from the typical *B. pullorum* by failure to form gas, but otherwise identical with it.

CHARACTERISTICS OF COLON-TYPHOID BACTERIA OF GROUP IV (FERMENTING MANNITOL, XYLOSE AND RHAMNOSE, GENERALLY PRODUCING GAS)

The fourth of the general groups into which we have divided the colon-typhoid series is that of which the B paratyphoids are the most typical examples. All of these forms ferment xylose and rhamnose and some of them arabinose as well, all produce a rather prompt alkalinity in milk, and all differ in agglutinative reactions from the A paratyphoid types. In general vigor of growth and fermentative power these forms stand nearer to *B. coli* than do any other of the non-lactose-fermenting organisms.

The first type of organism which we are inclined, with some doubt, to place in this group is the causative agent of fowl typhoid (*B. gallinarum*). This form, which was not represented in our own series, has the general fermentative reactions of the B. paratyphoids (see page 440) except that it attacks dextrin also, and that it fails to form gas. In view of the lack of correlation between gas production and power to attack various carbohydrates exhibited by the Morgan bacillus and the two varieties of *B. pullorum*, we are inclined to lay less stress on the former than on the latter characteristic. We cannot therefore agree with Hadley, Caldwell, Elkins and Lambert (1917) who believe that *B. gallinarum* is more closely allied to *B. typhosus* than is *B. pullorum*. All three organisms are however allied by their agglutinative reactions. According to Hadley, Elkins and Caldwell (1918) the milk reaction of *B. gallinarum* is of the B para-

typhoid type and the simultaneous fermentation of xylose and arabinose, a phenomenon which is so characteristic of paratyphoid B, seems to indicate that *B. gallinarum* may best be considered as a member of this group. It is certainly intermediate in its characteristics between *B. typhosus* and paratyphoid B; and it is of little consequence on which side of the arbitrary line between these types it may be placed.

The organism of fowl typhoid was described by Klein in 1889 as *B. gallinarum* and is probably identical with *B. sanguinarium* of Moore (1895). It may be characterized as follows.

B. gallinarum Klein. Gram-negative, non-spore-forming nonmotile rods. Forming colonies on gelatin somewhat intermediate between the thin translucent irregular colonies of *B. typhosus* and the regular convex colonies of *B. coli*. Ferments the hexoses, mannitol, maltose, arabinose, xylose, dulcitol, dextrin, rhamnose, sorbitol, but not adonitol, salicin, lactose, sucrose or raffinose. Forms acid but no gas. Milk, first acid, quickly turning alkaline and developing the translucent appearance characteristic of paratyphoid B. Does not liquefy gelatin or produce indol. Exhibits group agglutination reaction with *B. typhosus*. Found as causative agent in fowl typhoid.

Hadley, Elkins and Caldwell (1918) describe two allied species, *B. pfaffi* which differs from *B. gallinarum* in failing to ferment dextrin or dulcitol and in fermenting salicin; and *B. jeffersoni* which fails to ferment dulcitol and produces no change at all in milk.

B. avisepticus, the causative organism in fowl cholera, is a member of the Pasteurella group and does not belong in the colon typhoid series at all.

The more familiar organisms of the paratyphoid B group include, as pointed out above (p. 439) three distinct types, *B. suipestifer* (xylose + arabinose − inosite −), *B. enteritidis* (xylose + arabinose + inosite −), and paratyphoid B, itself (xylose + arabinose + inosite +).

Of the first of these types we had 5 strains in our collection, 3 of which were sent to us as *B. cholerae-suis*, one as *B. paratyphi*, and one as *B. sternbergii*. All fermented the hexoses, mannitol,

xylose and rhamnose but not arabinose, lactose, sucrose, salicin or inosite. Two attacked dulcitol and three did not. Milk was in all cases turned blue in two days and deepened in color progressively. Gelatin was not liquefied, indol was not produced, and lead acetate was not blackened. One strain of *B. cholerae-suis*, otherwise the same as the five mentioned, failed to ferment rhamnose.

Of the paratyphoid B forms which ferment arabinose as well as xylose and rhamnose, we had 24 strains in our collection. Five were originally sent in as paratyphoids, five as *B. enteritidis*, six as mouse and rat viruses of various sorts (*B. danysz*, *B. murium*, *B. murisepticus*), three as *B. abortivius*, and five under other names (*B. typhi-suis*, *B. typhosus*, *B. pullorum*, *B. icteroides*, *B. paracoli*). All were alike in fermenting the hexoses, mannitol, xylose, arabinose, and rhamnose and in failing to ferment lactose, salicin and sucrose. Dulcitol was attacked by nineteen strains and inosite by six (strains 22, 30, 169, 235, 237, 589). None formed indol or liquefied gelatin. Lead acetate was browned by all but the three strains of *B. abortivius*. Litmus milk was slightly reddened in two days but by the sixth day was always distinctly alkaline and became progressively more blue with a characteristic translucency.

These results obtained by us are in accord with the findings of previous students of this group, of whom the most important have been Boycott (1906), Bainbridge (1909), Harding and Ostenberg (1912), Jordan (1917), Krumwiede, Pratt and Kohn (1917), and Krumwiede, Kohn and Valentine (1918).

All observers agree that the paratyphoid organisms are motile. Boycott (1906), Smith (1915) and Jordan (1917) point out that they ferment maltose, and sorbitol; Jordan (1917) and Krumwiede, Kohn and Valentine (1918) report failure to attack raffinose, adonitol, dextrin, inulin and erythritol. The reduction of neutral red is described by Sacquépée and Chevrel (1906), Bainbridge (1909), and Hollande and Beauverie (1915). High resistance to green dye is reported by various observers.

Of the three species included in this group the first, distinguished by failure to attack arabinose and generally negative

results in dulcitol, is the organism isolated by Salmon and Smith from cases of hog cholera in 1885. This form has been confused with an organism of the hemorrhagic septicemia group associated with swine plague from which it is quite distinct. The type found in hog cholera should bear the name *B. suipestifer* and the swine plague organism that of *B. suisepticus*. *B. suipestifer* according to Jordan (1917) is a form quite constantly of porcine origin, while both *B. enteritidis* and paratyphoid B are commonly isolated from human sources. It is distinguished from the human forms by failure to brown lead acetate media and, according to Krumwiede, Kohn and Valentine (1918) by the power to reduce Andrade's indicator in glucose-serum-water. It may be defined as follows, culture 258 obtained from Parke Davis and Company in 1911 labelled "Boxmeyer, Belle Plain, 1903 No. 053" being taken as the type.

B. suipestifer Kruse. Gram negative, non-spore-forming, motile rods. Forming colonies on gelatin intermediate between the thin translucent irregular colonies of *B. typhosus* and the regular convex colonies of *B. coli*. Ferments the hexoses, mannitol, maltose, xylose, rhamnose and sorbitol with formation of gas. Does not ferment arabinose, lactose, sucrose, salicin, inosite, raffinose, adonitol, dextrin, inulin or erythritol. Generally fails to attack dulcitol. Turns milk first slightly acid, reverting in five days to an alkaline reaction, the color of the medium deepening with the development of a translucent appearance. Neutral red and malachite green reduced, lead acetate not blackened. Indol not produced. Gelatin not liquefied. Group agglutination with paratyphoid B. Found in intestines of hogs and as secondary invader of tissues in hog cholera.

B. enteritidis is distinguished from *B. suipestifer* by fermentation of arabinose, general fermentation of dulcitol, and blackening of lead acetate media, and from paratyphoid B by failure to ferment inosite. It exhibits distinct and characteristic serum agglutinative reactions. It may be defined as follows.

B. enteritidis Gaertner. Resembles *B. suipestifer* in all cultural characters except that arabinose is always, and dulcitol

generally fermented, and that lead acetate media are blackened. Exhibits characteristic serum agglutination reactions which distinguish it from either *B. suipestifer* or paratyphoid B. Found in human intestine and as causative factor in outbreaks of food poisoning. The type is our strain 18, a Gaertner strain obtained from the Rockefeller Institute in 1911.

Finally there remains for consideration the true paratyphoid B organism which is distinguished from *B. enteritidis* by the fermentation of inosite and by its serological reactions. So far as we are aware no one has ever given this very distinct type a specific name in proper Latin form; and since the identification of the paratyphoids merely as "A" and "B" is quite misleading as to their true biological relationships, we suggest the name *B. schottmulleri* for the paratyphoid B organism, to be defined as follows.

B. schottmulleri. Resembles *B. suipestifer* in all cultural characters except that arabinose and inosite are always fermented and dulcitol is generally fermented and that lead acetate media are browned. Its agglutinative reactions distinguish it from *B. enteritidis.* Found in human intestines and urine and as causative agent in paratyphoid fever and food poisoning outbreaks. The type is our strain 22, a Schottmüller strain obtained from the Rockefeller Institute in 1911.

The various forms of mouse and rat virus appear not to be distinct entities according to Bainbridge (1909), Savage (1912) and Krumwiede, Pratt and Kohn (1917); and our own results bear out their view that some of these viruses belong to each of the three species listed above, the Danyz virus being a variety of *B. enteritidis.*

The peculiar organism known as the Morgan bacillus may perhaps best be considered here, although its exact relationships are obscure, since it possesses the power of forming gas but can attack only a limited number of carbohydrates. Five of our cultures were of this type. Two of these strains came to the Museum as Morgan bacilli while the others were labeled respectively *B. communis, B. cuniculicida* and *B. pseudotuberculosis,* but all were alike in their failure to ferment the higher carbohydrates and in the production of a small but definite amount of

gas (about 5 per cent) in glucose, mannose, fructose and galactose. The acid production in the hexoses was vigorous in all these five strains when they were first tested in 1917, but when re-examined in 1919 one of them (strain 586) had lost its power to attack any of the sugars. This observation, together with the fact that one of the strains (strain 139) which attacked the hexoses, but only the hexoses, in both our tests was originally obtained from D. D. Jackson as *B. communis* A (fermenting mannitol, dulcite, lactose and raffinose) again suggests a loss of fermentative power during prolonged cultivation in the laboratory. In milk cultures all of the strains produced an immediate alkaline reaction, later decolorizing the litmus to some extent so as to produce a chalky blue appearance. This fact is probably associated with the rapid decomposition of protein characteristic of this form. Kendall, Day and Walker (1913) note a production of 4.2 mg. of NH_3 per 100 cc. of glucose broth for the Morgan bacillus in one day, as against less than 0.1 mgm. for *B. typhosus* and 0.2 for the paratyphoids. All of our strains formed indol and failed to liquefy gelatin.

Morgan (1906) describes this organism as motile and differentiates it from the hog cholera type by its reaction in litmus milk, active production of indol and failure to ferment maltose, arabinose and dextrin. Organisms of this type have been studied by Lewis (1912), Alexander (1912) and particularly by Graham-Smith (1912). The latter investigator prepared an elaborate system of classification of the non-lactose-fermenting bacteria, his type G including the forms which produce gas in glucose but not in mannitol media, being the most common of all the types in the lactose-negative group. Tribondeau and Fichet (1916), who isolated the Morgan bacillus from 13 cases of dysentery originating in the Dardanelles note, in addition to the characters described above, that it produces a fluorescence in neutral red broth and rapidly blackens lead acetate. They found several strains of related organisms which at first fermented maltose and sucrose but which later lost this power.

It is evident that the Morgan bacillus, wherever its closest relationships may lie, constitutes a fairly definite type, of com-

mon occurrence in the human intestinal canal. So far as we are aware it has nowhere been given a scientific name in proper form; and we have therefore called it *B. morgani*, characterizing the species as below and considering culture 692 of the American Museum collection as its type. This strain was isolated from the stool of an infant at the Providence City Hospital by H. E. Smiley.

B. morgani. Gram-negative, non-spore-forming motile rods, producing thin translucent irregular colonies on gelatin. Rapid formation of acid and slight gas production in media containing the hexoses. Milk turned gradually blue. Indol formation vigorous. Gelatin not liquefied. Produces fluorescence in neutral red broth and blackening in lead acetate media. Found in normal and diarrheal stools.

This organism would seem to be allied to *B. shigae* by its limited fermentative powers, to *B. dysenteriae* (or *B. coli*) by indol production, and to the paratyphoids by the formation of gas. It may perhaps represent an extreme variant of the variable *B. dysenteriae* group but we have considered it with the paratyphoids on account of its gas production.

One other type of non-lactose-fermenting organism often considered as a member of the colon-typhoid group is the *B. proteus;* but on account of the fundamental differences in morphology and metabolism (fermentation of sucrose but not lactose and very vigorous decomposition of proteins) we believe the Proteus forms should not form a part of this series at all.

CHARACTERISTICS OF COLON-TYPHOID BACTERIA OF GROUP V (FERMENTING LACTOSE AND THE SIMPLER CARBOHYDRATES WITH PRODUCTION OF EQUAL VOLUMES OF CO_2 AND H_2)

This group of organisms, of which *B. coli* is the most familiar example, is distinguished from all the organisms of the dysentery-typhoid-paratyphoid series by the power of fermenting lactose and from the *B. aerogenes* types by the fact that in its fermentation it produces equal volumes of CO_2 and H_2 (low ratio fermentation of Clark and Lubs). Its members differ from the

typhoid and paratyphoid organisms in less active motility, in more vigorous growth on media (with more convex and regular colonies), in strong reducing action, in the formation of indol, and in characteristic differences in resistance to various antiseptics.

In the Museum collection we found 42 different strains belonging to this general group. All of them fermented the hexoses, mannitol, xylose, arabinose, rhamnose, and lactose, with the production of rapid and permanent acidity and gas. None attacked inosite while with salicin, dulcitol and sucrose results were variable. All acidified and coagulated milk promptly, a firm clot being generally formed in six days with marked decolorization of the litmus. Indol was formed by all but 10 strains. Gelatin was not liquefied.

In regard to other reactions which we did not study it may be noted that maltose is fermented, according to Levine and other workers; that adonitol is generally not attacked (Mac Conkey, 1909, Rogers, Clark and Lubs 1918); and that starch, glycogen, inulin and dextrin give negative results according to Chantemesse and Widal (1891), Drigalski and Conradi (1902), Burk (1907), MacConkey (1909), Rogers, Clark and Evans (1914, 1915), and Levine (1916). Murray (1916) however reports that a fair proportion of strains from bovine feces do attack inulin.

Twort (1907) in a study of the utilization of a large series of unusual glucosides found that *B. coli* generally attacked euonymin, iridin, senegin, coniferin, arbutin, salicin, syringin, quillajinic acid, populin, camellin, and globularin. The hydrolytic splitting of esculin with the production of sugar and a substance called esculetin which reacts with iron citrate to produce a brown color has been used by Harrison and van der Leck (1909) and others as a test for this group. The colon bacillus generally reduces nitrates and neutral red (Rothberger, 1898); but does not brown lead acetate media (Sacquépée and Chevrel, 1905, Burnet and Weissenbach, 1915). It produces an acid reaction in Capaldi and Proskauer's medium 1 but not in medium 2. It has a relatively high resistance to acid (Hankin, 1899, Winslow and Lochredge, 1902) potassium tellurite (Davis, 1914), and cholesterin (Manfredi, 1917); but a relatively low resistance to

the green dyes (Loeffler, 1903, 1906, Lentz and Tietz, 1903, 1905, Krumwiede and Pratt, 1914), caffeine (Hoffman and Ficher, 1904), and bile salts (Jackson and Melia, 1909).

The further subdivision of the colon group of bacteria was first attempted on the basis of the fermentation of sucrose. Germano and Maurea (1893) for example distinguished one type which fermented sucrose and decolorized jequrity solution and another which failed to give either of these reactions. Smith (1893, 1895a) in his classic investigations emphasized the importance of the sucrose-positive and sucrose-negative varieties. Durham (1901) gave the sucrose-positive form the name *B. communior*. Winslow and Walker (1907) and Howe (1912) pointed out that the forms which attack sucrose also attack raffinose and vice versa.

Very elaborate classifications of the colon group were developed by MacConkey (1905), Bergey and Deehan (1908), and Jackson (1911), based primarily on fermentation of sucrose and dulcitol and secondarily on fermentation of adonitol and inulin, the Voges-Proskauer reaction, motility, indol formation and liquefaction of gelatin. The principal types recognized by these authors were as follows.

I. Sucrose − Dulcitol − *B. acidi-lactici*
II. Sucrose − Dulcitol + *B. coli-communis*
III. Sucrose + Dulcitol − *B. coscoroba* [*B. aerogenes* (V. − P. +), and *B. cloacae* (V. − P. +, Gelatin +) also belong here]
IV. Sucrose + Dulcitol + *B. communior* [*B. neapolitanus* (non-motile) and *B. pneumoniae* also belong here]

These classifications were all defective in grouping the Voges-Proskauer positive, indol negative, gelatin positive organisms with the various fermentative types of the true colon group. We now know that these forms belong to the high ratio (*B. aerogenes*) group and should not be placed with *B. coli* and *B. communior*. *B. acidi-lactici*, *B. coli*, *B. coscoroba* and *B. communior* may however be considered to represent distinct types, if the fermentation of dulcitol is really a characteristic of specific importance. There seems grave doubt however whether this

alcohol should be given a prominent place in classification; for as pointed out above dulcitol throughout the colon-typhoid series shows less correlation with other properties than any other carbohydrate and under experimental conditions shows the most marked tendency to spontaneous variations.

Rogers, Clark and Evans (1914), in a study of colon organisms from bovine feces, found that sucrose and raffiinose fermentations are directly and almost perfectly correlated, and that the sucrose positive forms are generally dulcitol positive and adonitol negative, while the sucrose negative forms vary in both dulcitol and adonitol. The sucrose negative forms may be divided (although the authors do not point this out) into a dulcitol + adonitol − group and a dulcitol − adonitol + group. Kligler (1914) emphasized the value of salicin as a differential test substance, finding dulcitol inversely correlated with salicin among the sucrose positive forms and positively correlated among the sucrose negative forms. Failure to attack glycerol was most common among the salicin-fermenters.

The most extensive study of this group of organisms is that conducted by Levine (1917). This investigator worked with 333 strains isolated from soil, sewage and the feces of men, horses, sheep, pigs and cows and devoted special attention to the mutual correlation of the various characters studied. One hundred and eighty-two of the strains were of the methyl-red-positive, Voges-Proskauer-negative type. Sucrose and raffinose were almost perfectly correlated and the author divides his methyl red positive organisms first on the sucrose fermentation. The sucrose negative forms are next subdivided according to their action on salicin, giving *B. coli* (sucrose − salicin +) and *B. acidi-lactici* (sucrose − salicin −) as species. Among the sucrose positive strains the most natural division, according to correlated characters, is between a motile type *B. communior* and a non-motile series which can be further subdivided into a salicin positive species *B. neapolitanus* and a salicin negative species, *B. coscoroba*. *B. communior*, *B. neapolitanus* and *B. coscoroba* were much more common in animal than in human feces, while *B. coli* and *B. acidi-lactici* were commonly isolated from human sources.

It is impossible to make a direct and comprehensive comparison of the results of these various investigators since each one used some tests not applied by the others. In our own investigations we did not study motility or the fermentation of adonitol and salicin. The following characteristics would appear to be indicated for the principal types recognized by Kligler and Levine.

	SUCROSE	SALICIN	DULCITOL	ADONITOL	MOTILITY
B. neapolitanus	+	+	−	−	−
B. communior	+	−	+	−	+
B. coscoroba	+	−	+	−	−
B. coli-communis	−	+	+	−	+
B. immobilis	−	+	+	−	−
B. Grünthal	−	−	−	+	+
B. acidi-lactici	−	−	−	+	−

All recent observers are agreed as to the superior value of salicin as compared with dulcitol for the primary subdivision of this group; and Levine's main classification, based on so large a series of strains can safely be accepted as in the main a correct one. We cannot feel certain however that the presence or absence of motility is a sufficient basis for the establishment of species, in view of the highly inconstant results obtained by other students of this property. We are therefore inclined to recognize four distinct species, as characterized below, with three varieties, based on motility.

B. neapolitanus Fraenkel. Gram-negative non-spore-forming rod. Non-motile. Vigorous growth on media, colonies more regular and convex than those of *B. typhosus*. Produces prompt and permanent acidity and gas composed of equal volumes of CO_2 and H_2 in media containing the following substances; the hexoses, maltose, mannitol, xylose, arabinose rhamnose, lactose, sucrose, salicin and esculin, but not as a rule dulcitol or adonitol, and never inosite, starch, glycogen, inulin or dextrin. Turns milk strongly acid and coagulates it in six days at 37°. Generally forms indol but does not liquefy gelatin. Produces an acid reaction in Capaldi-Proskauer medium 1 but

not in medium 2. Does not give Voges-Proskauer reaction. Reduces neutral red and generally nitrates. Does not brown lead acetate media. Exhibits high tolerance to acids, potassium tellurite and cholesterin, but relatively low tolerance to the green dyes, alkaloids and bile salts. Found in feces of higher animals and man, particularly the former.

Our type of this species is strain 126, isolated from a urinary fistula in 1911 and received by us from Dr. Jackson of the Mt. Prospect laboratory as *B. aerogenes* A2.

B. communior Durham. Differs from *B. neapolitanus* in failing to ferment salicin, in generally fermenting dulcitol and in being sluggishly motile. Our type is 137 isolated from feces by Dr. Frazer in 1911 and sent to us by Jackson as *B. communior.*

var. *coscoroba,* differs from *B. communior* in lacking motility.

B. coli Escherich. Differs from *B. neapolitanus* as described above in failing to ferment sucrose and raffinose, in generally fermenting dulcitol, and in exhibiting sluggish motility. Specially abundant in human feces. Type, 125 isolated from a case of cystitis in 1910 and sent to us by Jackson as *B. communis B.*

var. *immobilis* differs from *B. coli* in being non-motile.

B. acidi-lactici Grotenfelt. Differs from B. neapolitanus in failing to ferment sucrose, raffinose or salicin and in generally fermenting adonitol. Specially abundant in human feces. Type, 131 from Jackson as *B. acidi-lactici B.* Isolated from feces.

var. *Grünthal.* Differs from *B. acidi-lactici* in being motile.

All of the type specimens listed above are described by Jackson (1911). He states that type 137 does not ferment mannitol but we find that it does.

The organisms in our collection did not show by any means a clear cut correlation between the fermentation of sucrose, salicin and dulcitol. Classifying them according to their action upon sucrose and salicin we found 15 strains of *B. neapolitanus* (of which only 5 were negative in dulcitol), 4 of *B. communior* (of which 3 were negative in dulcitol), 16 of *B. coli* (of which 10 were positive in dulcitol), and 7 of *B. acidi-lactici* (of which 4 were negative in dulcitol). The names under which these organisms were sent to us corresponded fairly well with the species as

identified except that the *B. neapolitanus* series included two strains called *B. aerogenes*, one *B. pneumoniae*, and one *B. ozenae*, one *B. sternbergii* and one *B. bovisepticus;* the *B. communior* series one *B. bronchicanis;* the *B. coli* series, a *B. diphtheriae-columbarum*, two *B. cholerae*, one *B. astheniae*, one *B. anaerogenes*, one *B. aerogenes*, and 3 *B. acidi-lactici;* and the *B. acidi-lactici* series two *B. coli*, one *B. voldagsen*, one *B. danysz*, and one *B. dysenteriae* (!).

CHARACTERISTICS OF COLON TYPHOID BACTERIA OF GROUP VI (FERMENTING LACTOSE AND THE SIMPLER CARBOHYDRATES WITH PRODUCTION OF TWO OR MORE VOLUMES OF CO_2 TO ONE OF H_2)

The last group of the colon-typhoid series, of which *B. aerogenes* and *B. cloacae* are the principal types, was recognized by Escherich (1885) on the basis of greater plumpness of the cell form, lack of motility and more rapid coagulation of milk, by Smith (1893a) on the basis of heavier growth and tendency to capsulation, and later (Smith 1895a) on rapid gas production and higher ratio of CO_2 to H_2. Durham (1901) notes the fermentation of starch and inulin and the Voges-Proskauer reaction as characteristic, Grimbert and Le Gros (1900) and Jordan (1903) the failure to form indol. Finally Harden and Walpole (1905) and Rogers, Clark and Davis (1914) demonstrated conclusively the fundamental difference in carbohydrate metabolism between these forms and those of the *B. coli* type. Rettger (1903) showed that the differences in protein metabolism are equally distinct, *B. aerogenes* being much less active in this respect forming mercaptans, skatol, phenols, aromatic oxy-acids and skatol-carbolic acid much more slowly. Ferreira, Horta and Paredes (1908) report that *B. aerogenes* and *B. cloacae* ferment sucrose but not dulcitol and give a rose color with no luster on the Endo medium. Rivas (1908) points out that *B. aerogenes* exhausts the carbohydrate constituent in glucose broth much more rapidly than does *B. coli.* Rogers, Clark and Davis (1914) described a series of high gas ratio cultures isolated

from milk, and pointed out that these forms attack sucrose, raffinose, and starch and liquefy gelatin more frequently than do the *B. coli* forms, and on the other hand attack dulcitol and glycerol less often. Rogers, Clark and Evans (1915) studied 166 high ratio cultures from grains and described six different types of which the most abundant had the following characteristics.

Gelatin-liquefying forms. Not capsulated, indol negative, sucrose positive, raffinose positive, mannitol positive, dulcitol positive, glycerol generally positive, starch negative, inulin negative, adonitol negative.

Gelatin-non-liquefying forms. Sometimes capsulated, sometimes fermenting starch, inulin and adonitol, rarely fermenting mannitol or dulcitol, otherwise as above.

Rogers, Clark and Lubs (1918) describe a series of high ratio strains from human feces as generally indol negative, sucrose, raffinose, mannitol and adonitol positive, inulin negative, and generally dulcitol negative. According to these results the high ratio type common in human feces differs from that normal on grains by the fermentation of mannitol and adonitol.

Levine (1916b) states that *B. aerogenes* usually ferments sucrose, raffinose, salicin, glycerol, dextrin and starch, but rarely dulcitol. In a later paper (Levine 1917) he confirms the conclusion of Kligler (1914) (opposed to that of Rogers, Clark and Evans) that gelatin liquefaction and glycerol fermentation are negatively correlated. Of 151 strains of the high ratio type (isolated from soil or in a few cases from sewage) all fermented mannitol, and almost all sucrose, raffinose, and salicin. He recognizes two species, *B. aerogenes*, which rarely liquefies gelatine, is non-motile and forms gas from glycerol and starch; and *B. cloacae* which is a liquefying organism failing to ferment either glycerol or starch.

In general it is evident that the organisms of this group may be subdivided into a type which liquefies gelatin and one which fails to do so. The former, *B. cloacae*, is not capsulated, and does not ferment starch, inulin or adonitol, while *B. aerogenes* is often capsulated and sometimes ferments starch and inulin

and adonitol. According to Rogers, Clark and Lubs the grain type of *B. aerogenes* differs from the fecal type in its failure to attack mannitol and dulcitol. Twort (1907) reports the frequent fermentation by *B. aerogenes* of a number of glucosides not attacked by *B. coli* such as cerberid, periplocin, cathartinic acid, amygdalin, sapotoxin, saponin, bryanin, convallamarin, digitalin, strophanthin, coronillin, gratiolin and phloridzin.

Our own series of cultures included 8 of the *B. cloacae* and 23 of the *B. aerogenes* type. All gave positive results in the hexoses, mannitol, xylose, arabinose, rhamnose, lactose, salicin and sucrose. All coagulated and decolorized milk, the clot being formed by 19 strains in six days at 37°, by the other 12 more slowly. Only 6 strains formed indol. All but 1 were alkaline to methyl red and all but 8 gave the Voges-Proskauer reaction. Only 9 fermented dulcitol and 16 fermented inosite. A special study showed that our gelatin-liquefying strains all failed to ferment glycerol, while the *B. aerogenes* did attack this substance.

We may therefore recognize at least two species in this group, as follows.

B. cloacae Jordan. Gram-negative non-spore-forming rod. Non-motile. Vigorous growth on media, colonies even more regular and convex than those of *B. coli*. Ferments the hexoses, maltose, mannitol, xylose, arabinose, lactose, rhamnose, sucrose, raffinose, and salicin, often inosite, but generally not dulcitol, glycerol, starch, inulin or adonitol. Produces vigorous and complete destruction of the carbohydrates which it attacks forming acetyl-methyl-carbinol (Voges-Proskauer reaction) and at least twice as much CO_2 as H_2, the reaction of the medium becoming moderately acid and then reverting to a lower degree of acidity. Acidifies and coagulates milk but not quite so promptly as *B. coli*. Fails to form indol. Liquefies gelatin slowly. Found in human and animal feces, sewage and soil. Our type of this species is type 23 isolated by Jordan from the Chicago Drainage canal in 1899.

B. aerogenes Escherich. Gram-negative non-spore-forming rod. Generally non-motile. Frequently capsulated. Growth on media vigorous, colonies convex and often viscid in texture.

Ferments the hexoses, maltose, xylose, arabinose, rhamnose, lactose, sucrose, raffinose, salicin, usually glycerol, starch, and inulin, and sometimes inosite and adonitol, dulcitol and mannitol. Produces vigorous and complete destruction of the carbohydrates, forming acetyl-methyl-carbinol (Voges-Proskauer reaction), and at least twice as much CO_2 as H_2, the reaction of the medium becoming moderately acid and then reverting to a lower degree of acidity. Acidifies and coagulates milk but not quite so promptly as *B. coli*. Fails to form indol or liquefy gelatin. Found in human and animal feces and sewage but particularly in soil and on grains.

Our type strain is type 240 received from Johns Hopkins in 1911 with following history—"probably a descendant of the original capsule bacillus of Pfeiffer . . . . reaction identical with *B. aerogenes*."

A variety which fails to ferment mannitol and adonitol is the commonest form on grains.

B. aerogenes represents the extreme of fermentative power in the colon-typhoid series; and on the assumption that the course of evolution has been marked by progressive loss of fermentative power and acquisition of the parasitic habit it may be considered the most primitive type of the whole group. It has been suggested by one of us (Kligler, 1917) that three different lines of evolution may have started from this type, the first leading through capsulated streptococci to the diverse forms of streptococci and pneumococci of the present day, the second through *B. coli*, *B. typhosus* and the dysentery organisms to the hemorrhagic septicemia group, and the third through *B. cloacae* and *B. proteus* to the saprophytic spore-bearing and pigment bacteria.

There is one group of organisms usually considered to be related to *B. aerogenes* whose affiliations appear to be obscure. These are the capsulated forms frequently met with in association with certain pathological conditions. Capsulated bacilli were first described by Friedlander from pneumonia in 1883; and important studies of this group have been made by Fricke (1896), Strong (1899), Perkins (1904) and Coulter (1917).

All of these workers assumed that the pathogenic capsulated bacilli with which they worked (*B. pneumoniae*, *B. ozenae*, *B. rhinoscleromatis*) were related to *B. aerogenes;* but all agree that they differ fron *B. aerogenes* in producing a more distinct capsule and colonies of a translucent syrupy consistency; in forming indol; and in fermenting sucrose but not lactose. The last property is a very rare one in the colon-typhoid group, although the fermentation of sucrose but not lactose is characteristic of the Proteus bacilli. In our Museum collection we had a number of organisms which were sent to us bearing the names of the capsulated pathogenic forms; but none of them exhibited the characteristics described by Perkins, Strong and Coulter. Of three cultures sent to us as *B. pneumoniae* one was finally classed as *B. alcaligenes*, one as *B. neapolitanus*, and one as *B. aerogenes*. Of two sent in as *B. ozenae*, one was *B. alcaligenes* and one *B. neapolitanus*. Two sent in as *B. capsulatus* and one as *B. rhinoscleromatis* all proved to be *B. aerogenes*. Thus of eight strains originally believed to be members of this group, four were of the methyl red negative, Voges-Proskauer positive type, while two belonged at the other extreme of the paratyphoid series, with no fermentative powers at all. It seems evident, either that we are dealing with an extraordinarily variable group or that forms which are not really related have been identified as of this type merely because of the possession of a capsule. (Fletcher, 1918, has recently described capsulated forms of paratyphoid and dysentery bacilli). The evidence brought forward by Strong (1899), Perkins (1904) and Coulter (1917) is so completely concordant as to make it clear that there is a distinct type of capsulated bacillus which ferments sucrose and not lactose and possesses considerable pathogenic powers; but in view of the incompleteness of our knowledge of its other reactions we are somewhat uncertain of its relations to the colon-typhoid group.

REVIEW OF THE GENERAL CHARACTERISTICS OF THE SPECIES OF THE COLON-TYPHOID GROUP

The principal characters of the species of colon-typhoid bacteria as defined above are presented for convenient comparison in the table below, and the fermentative relationships of certain of the more important forms are presented in figure I.

If *B. aerogenes* be taken as a representative of the most primitive type it is evident that there is a more or less steady decrease

SPECIES	HEXOSES	MALTOSE	MANNITOL	XYLOSE	ARABINOSE	RHAMNOSE	SORBITOL	DULCITOL	LACTOSE	SALICIN	SUCROSE	RAFFINOSE	INOSITE	DEXTRIN	GAS	VOGES-PROSKAUER	METHYL RED	MILK	GELATIN	INDOL	LEAD ACETATE	MOTILITY	PATHOGENIC
B. aerogenes	+	+	±	+	+	+	+	∓	+	+	+	+	+	+	+	+	−	Coag.	−	−	−	−	−
B. cloacae	+	+	+	+	+	+	+	∓	+	+	+	+	±	±	+	+	−	Coag.	+	−	−	−	−
B. neapolitanus	+	+	+	+	+	+	+	∓	+	+	+	+	−	−	+	−	+	Coag.	−	+	−	±	−
B. communior	+	+	+	+	+	+	+	±	+	−	+	+	−	−	+	−	+	Coag.	−	+	−	±	−
B. coli	+	+	+	+	+	+	+	±	+	+	−	−	−	−	+	−	+	Coag.	−	+	−	±	−
B. acidi-lactici	+	+	+	+	+	+	+	∓	+	−	−	−	−	−	+	−	+	Coag.	−	+	−	∓	−
B. morgani	+	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	+	al.	−	+	+	+	+
B. schottmulleri	+	+	+	+	+	+	+	±	−	−	−	−	+	−	+	−	+	ac. al.	−	−	+	+	+
B. enteritidis	+	+	+	+	+	+	+	±	−	−	−	−	−	−	+	−	+	ac. al.	−	−	+	+	+
B. suipestifer	+	+	+	+	−	+	+	∓	−	−	−	−	−	−	+	−	+	ac. al.	−	−	−	+	+
B. gallinarum	+	+	+	+	+	+	+	+	−	−	−	−	−	+	−	−	+	ac. al.	−	−	−	−	+
B. pullorum	+	−	+	−	+	+	+	−	−	−	−	−	−	−	−	−	+	ac. al.	−	−	−	−	+
B. paratyphosus	+	+	+	−	+	+	+	±	−	−	−	−	−	−	+	−	+	ac. al.	−	−	−	+	+
B. typhosus	+	+	+	+	−	−	+	−	−	−	−	−	−	+	−	−	+	ac. al.	−	−	+	+	+
B. dysenteriae	+	±	+	∓	±	∓	±	∓	−	∓	∓	∓	±	±	−	−	+	ac. al.	−	±	−	−	+
B. shigae	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	ac. al.	−	−	−	−	+
B. alcaligenes	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	al.	−	−	+	+	−

of fermentative power, down through the colon, paratyphoid and typhoid groups to *B. shigae* and *B. alcaligenes*. It cannot however be mantained that this progression necessarily or even probably represents the exact line of evolutionary development. In certain respects (as in its action on lead acetate media) *B. typhosus* seems more closely allied to *B. schottmulleri* than to *B. paratyphosus*. *B. dysenteriae* as noted exhibits highly variable reactions on maltose, xylose, arabinose, dextrin, rhamnose,

salicin and sucrose, which have therefore been left out of the chart entirely. *B. morgani* is another highly variable and unstable type, as is also *B. pullorum*; while the capsulated pathogenic forms allied to the Friedlander bacillus form an exceedingly puzzling complex. On the other hand *B. shigae* and *B. typhosus*

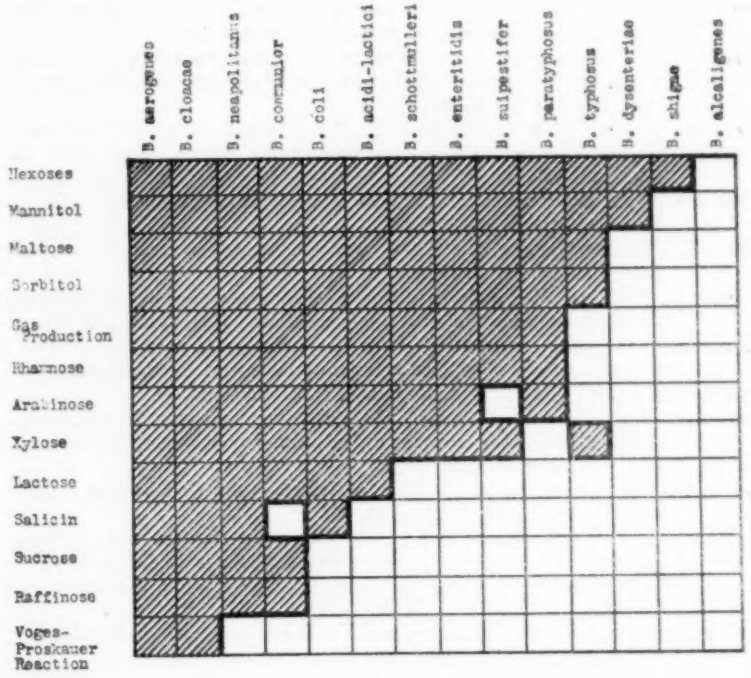


FIG. I

are species of very definite and constant characteristics; while *B. schottmulleri*, *B. enteritidis*, *B. suipestifer*, and *B. paratyphosus*, as well as the chief colon-aerogenes types, can be identified with reasonable ease.

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A CONTRIBUTION TO THE BACTERIOLOGY OF A FUSO-SPIRILLARY ORGANISM, WITH SPECIAL REFERENCE TO ITS LIFE HISTORY[1]

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This study has as its origin an obscure clinical case of generalized infection with a fuso-spirillary organism. In this communication I do not deem it desirable to allocate its place in the scale of organic life with finality, partly because of present classificatory readjustments that are being carried out by the Society of American Bacteriologists, and partly because of additional work that we are prosecuting with these and related organisms. The term fusospirillary I shall employ generically, although by some it has been temporarily preempted for the fusiform bacillus and the so-called spirochaetes found in Vincent's Angina. This disease and many other related ones will fall within the scope of the series of studies being at present conducted, which it is hoped will prove advantageous to a more fundamental understanding of their bacteriology.

CASE HISTORY AND SOURCE OF THE CULTURE

In the summer of 1916, J. B., sixteen years of age, underwent an operation for appendicitis. The latter condition was found to be complicated with an abscess, which was drained. Convalescence occupied a period of about six weeks. Although the wound healed, the patient did not regain his health, as was manifested by a continuous fever of

[1] This paper was presented before a joint session of the American Association of Immunologists and the American Society of Pathologists at their Annual Meeting, March 28-31, 1917, in Philadelphia.

from 100° to 102°. In March, 1917, a futile exploratory abdominal operation was performed, the symptoms directing attention particularly to the right hypochondriac region. The temperature fell to normal for a few days, following the operation, but soon rose to 103°–105°, after which very marked daily variation became the rule. In the morning it was usually normal and at times subnormal, only to be followed by a marked afternoon rise of 103°–105°. At this time pus and albumin were found in the urine, a polymorphic leukocytosis of 17,000 to 20,000 was present, and a bronchial catarrh, which had been noticeable from the first, assumed a more severe character.

Prolonged search of the mucopurulent sputum revealed a few small, opaque, white spherules about 1 millimetre in diameter. They were not at all caseous, but were quite cohesive. They reminded one of the sulphur granules of actinomyces infection. The sputum was rich in lymphocytes. When the spherules were crushed they were found to consist of necrotic masses and a thread-like branching micro-organism apparently in pure culture. These organisms were non-acid fast and Gram-negative. Several months later samples of sputum showed the same cohesive granules to consist of interlacing, very slender filamentous forms which were made up apparently of single rows of tiny granules. They stained poorly in Loeffler's, but very well in fuchsin. Among them were found also some vibrio forms and fusiforms.

On March 28, 1917, a lung puncture was made at the ninth right interspace, in the post-axillary line, at the point of greatest tenderness. A slight amount of serum and flaky material was obtained, resembling the spherules found in the sputum. Examination of the material showed it to consist almost entirely of bacterial growth. There was marked pleomorphism of the elements found. Asymetric bacillary or filamentous forms combined with large circular or ovoid forms predominated. The former varied from 5 to 20 microns long, the latter from 2 to 4 microns in diameter. These circular forms simulate the large giant cocci to be described in the first blood culture. There were also many thin wavy filamentous forms. These organisms grew only anaerobically. In broth the circular forms gradually disappeared leaving filamentous and coccal forms. The latter varied in size from about 0.25 microns to 1 or 2 microns. They usually occurred in groups. Repeated anaerobic plating showed that this culture was probably pure despite the confusing variety of forms.

On April 7, 1917, a second abdominal operation was performed, revealing a renal abscess, the pus from which was aspirated and a piece

of the kidney removed for histologic examination. The pus contained curved bacillary forms, occasionally faintly granular and not infrequently branching. They were frankly Gram-negative and in Loeffler's stained poorly. In Romanowsky they stained deeply blue. There was also present an occasional small group of metachromatically staining, extremely small coccal and diplococcal forms. This pus yielded absolutely no growth on any medium under aerobic conditions. On blood agar, anaerobically after nine days, small elevated colonies appeared. They had precisely the morphology of the Klebs-Loeffler bacillus, but emitted a putrid odor characteristic of *Bacillus fusiformis*. After three days the same pus on hydrocele agar developed greyish elevated colonies of a granular bacillus with pointed ends, which also developed this putrid odor (fig. 1). They had the staining reactions of *B. fusiformis*.

These organisms differ somewhat from the fusiform bacilli usually seen in exudates. Although many have the chromatin disposed as a single granule or as two granules paracentrally located, others show it disposed in rod shape, resembling vibrios. The latter may be S-shaped or straight. This culture stained in Romanowsky showed a delicate faintly staining substance surrounding the bacteria, in mantle-like fashion. It is in reality a periplast, which probably originates from the centrally located vibrio-like chromatin. Forms simulating these have been grown in association with the typical filamentous form and will be discussed further on page 522 (see fig. 2a).

The architecture of the kidney was almost entirely displaced by granulomatous tissue, characterized by fibrous change, and a diffuse infiltration with a large mononuclear type of cell answering the description of the endothelial leucocyte. In many places the infiltration seemed to be made up almost entirely of these cells. Typical plasma cells and polymorphonuclears were also present, the latter being most conspicuous in the remnants of the uriniferous tubules and glomeruli. The histopathology was suggestive of the actinomycotic lesion, but lacked the characteristic colonies. An examination for tubercle bacilli was negative, but a Gram-Weigert stain, cautiously decolorized, revealed most of the forms found in the smears of pus.

On December 7, 1917, one week before the patient's death, he developed multiple pleural sinuses from both pleural cavities. A sample of pus from each side stained in Romanowsky shows the variety of forms already described. There were large numbers of true fusiform bacilli, the giant coccus forms with peripheral granules, the small oval eccen-

trically granulated forms of various staining intensities, and the very thin wavy pink staining forms, some of which can be seen springing from the end of thicker, blue staining vibrio forms. There are also found the interrupted forms—slender curved bacilli in threads but connected by a non-staining link of appreciable length. All the discharges had the characteristic putrid odor of infections with *B. fusiformis* and the lesions were of a sloughing, gangrenous character, as was instanced by the breaking away of the wound stitches, due entirely to the direct but indolent extension of the process into the healthy tissue that held them. All attempts to obtain an autopsy on this patient were unfortunately futile.

The wide morphologic diversity evidenced by both the tissues and cultures favors the interpretation of a mixed infection, yet a careful analysis of the findings, especially the results of the blood cultures, revealed phenomena very inadequately explained by this hypothesis, and led me to seek a more satisfactory one. In the first place, the anaerobic character of all flora obtained from closed lesions (except the blood) was to say the least unusual, in consideration of the marked pleomorphism of the flora of the individual lesion. Most of the forms found were known to be rather closely related biologically. It is of note that the odor permeating the patient's room following the abdominal operation could be compared best with one thing, namely, a culture of *B. fusiformis*. Likewise the culture of *B. fusiformis* isolated from the renal pus as well as the aerobic coccus forms growing from the blood and from the Berkefeld's filtrates of the blood-serum produced this same odor, but the aerobic cultures lost it after the third generation. The fact that the aerobic coccus and the anaerobic bacillary form possessed this biologic character in common also suggested relationship between them.

The clinical course of the disease was akin to that of tuberculosis, or some mycosis. The intermittency of the clinical symptoms was most noteworthy, the patient having experienced about half a dozen clinical cures before he actually died. For weeks at a time he would have no fever, and would gain greatly in weight and strength. Assuming that the condition was mycotic in nature, it seemed reasonable to consider the various

morphological forms as phases in the life history of one organism. Such an hypothesis was particularly serviceable in the interpretation of a very peculiar, and in my experience novel phenomenon, observed in connection with the blood cultures. In its explanation the mixed infection idea was inadequate, and its investigation forms the starting point for the entire study.

BLOOD CULTURES

Ten cubic centimeters of blood were first grown in 50 cc. of broth in two separate flasks of the same medium (broth 8). After forty-eight hours a small amount of granular sediment appeared, but the supernatant was entirely clear. Examination of one flask at this time showed some very large circular forms (giant cocci, fig. 3) varying from 3 to 4 microns, staining faintly or not at all in Loeffler's and negative to Gram, except some granules at the periphery of the cell.[2] Wright's stain as well as cresyl blue were used to differentiate these from erythrocytes or their products. With Romanowsky they stained quite satisfactorily.

Until the fifth day the growth consisted of a slowly increasing, coarsely granular sediment. At this time, however, reproduction occurred with such amazing rapidity that the culture became diffusely clouded in three hours' time, which event constitutes the novel phenomenon to which I have referred. To my surprise the culture then contained a preponderance of usual-sized diplococcus and coccoid forms. The same findings were

[2] At this juncture 2 cc. of the broth (no. 8) were pipetted off this flask and placed at room temperature for a day and then in the ice-chest. In three days, long branching filaments developed, some taking their origin from the intracellular coccoids, others apparently arising from the peripheral granules of the giant cocci (figs. 4. 5, and 6). This culture has been carried for more than 100 generations under varying conditions, but diplococci such as germinated in the flask on the fifth day have never been separated from it. As will be seen, steps were taken to rule out contamination in this instance, and such response to environment is one of many observations favoring the cyclic nature of the changes seen. The cocci developing from the blood in another batch of broth, although suggesting filaments by the short spicules that arose from some of them, would without other evidence have been misleading.

present in the second flask which had not been exposed to the air by examination of it. A second blood culture taken in another flask of a different batch of broth (no. 16) developed these same diplococci and coccoid forms in twenty-four hours. Reference to the media book showed certain irregularities in the preparation of the first batch of broth. In order to ascertain the possible influence of this factor, a third culture of 10 cc. was taken and equally distributed between flasks of broths 8 and 16 with results which are a repetition of those just related. It seemed certain that the broths were quite different, and certainly possible that this difference found expression in the way just related.

Warm stage studies, the results of which will be described presently, suggested that the organism was present in the blood serum in filtrable form. Accordingly 5 cc. of freshly drawn citrated serum was diluted with 25 cc. of sterile NaCl solution, and passed through a Berkefeld N. filter. The filtrate was mixed with the no. 16 broth with the consequent development of the same diplococcus and coccoid forms found before. The luxuriantly growing diplococci were found pure on plating. Further evidence for the existence of the organism in the blood in filtrable form is seen in the fact that a suspension of these diplococci, grown as such, refused to pass the same Berkefeld filter that had previously passed the filtrable stage of the organism through its pores. Furthermore, the identical filter, after being cleaned and sterilized, once more passed the original sample of diluted blood serum and gave rise to the same cocci. The experiment was repeated in order to be sure that the refusal of the cultured diplococci to pass the filter was in no way dependent on a mechanical plugging of its pores during the first experiment. These experiments would seem to show that there existed in the blood serum filtrable forms which germinated into diplococci and coccoids, which forms themselves were not filtrable. But I wish to emphasize the fact that the experiments do not prove that these diplococci and coccoids may not give rise to another order of filtrable body, conditions for whose germination have not been fulfilled. The evidence for the exist-

ence of different orders of gonidia will be discussed later. The filtrable bodies I shall hereafter speak of as gonidia. Entirely in line with their presence in the blood is their microscopic appearance in the pus of the renal abscess. Stained in Romanowsky, they appear as groups of very small pyriform structures metachromatically staining. They are also found, together with the coccus and coccoid forms, in the pus as well as in the tissue of the renal granuloma itself.

A more detailed study of the provisionally designated giant cocci, already referred to as occurring in the blood culture, shows that some of them contain from four to six distinct oval intracellular bodies, similar in size and morphology with extracellular forms of the same size (fig. 5). These latter constitute part of the coccoid forms and are to be distinguished from the typical diplococci and the gonidia. In addition to the oval intracellular bodies, very many of the giant cocci average four chromatin granules, located usually at the poles of the cell.

This latter form has occurred frequently in generations far removed in number from the original blood culture. With Romanowsky the granules stained a deep blue, and were usually clearly defined, while the remainder of the cell was either achromic or varied from light pink in the younger forms to a magenta or frank blue in the older ones (figs. 4 and 7). This staining intensity increased first in the periphery of the cell and was often an indication that the filamentous phase of the organism had been reached. In fact, it can be shown that this peripheral staining intensity often appears to represent the development of intercommunicating filaments between the granules, which later may encroach on the central pink portion until ultimately the whole cell is intensely blue (fig. 4b). Under certain conditions the peripheral granules may develop lateral projections, resulting in a stellate form (fig. 4a). The cell body appears to disintegrate later, leaving the pleomorphic, deeply stained granules attached to long wavy filaments. These later may break up into vibrio and then small coccus forms. Under appropriate conditions, these coccus forms seem to fuse into an amorphous, granular mass, and giant cocci, having the character of many of those

found in the blood cultures will again appear, and the cycle start anew. In fact, the observations on the living forms, as detailed below, were seen in a transplant from such a culture.

WARM STAGE OBSERVATIONS

This method of study was adopted as constituting one of the most direct avenues for the solution of the problem created by the fulminating rapidity which characterized the advent of diplococcus forms into the broth culture. Figures 8 to 11 inclusive show that the giant cocci develop quite definite intracellular changes, the end result of which is the formation and liberation of gonidia. Inasmuch as this entire change occurred in less than half an hour, it might serve to explain the phenomenon above referred to. It seems probable, not only from direct observation but also from the study of stained specimens, that the oval intracellular forms, having on their central end the suggestion of a filament, gave rise to the extracellular forms by a process resembling schizogony among the protozoa.

It is important for a study of these forms that cultivation be carried out in serum or hydrocele broth, as the forms develop slightly if at all on solid media. My preparations were made by pipetting off some of the sediment to a perfectly clean slide, and covering with a cover glass, scrupulously clean, in such manner that no bubbles of air were enclosed, and that no excess of liquid projected from the edges of the cover slip. An airtight cell can then be made by rimming the edges with paraffin, and the preparation usually lasts at least twenty-four hours, if properly made. This is an old and well known method and for this purpose is much superior to the hanging drop; unless the preparation is free from air bubbles, however, much difficulty will be experienced in the necessary stability of the object to be studied. It should not be imagined that these changes can be seen on any occasion or under any conditions. One may search many hours, even days, in properly prepared specimens containing many large forms, before observing clean-cut and definite changes. And when they do occur, they may be of an abortive nature, as indeed happened twice in the specimen described, before the actual cycle depicted was finally consummated.

FIG. 8A

FIG. 8B

FIG. 8A. GIANT COCCUS MEASURING FROM 3½ TO 4μ AS SEEN AT 9.00 A. M.

The highly refractile, finely granular central body changes location rapidly, and is actively amoeboid.

FIG. 8B. REPRESENTS THE IRREGULAR, STRUCTURELESS FORM OR RESTING STAGE, FOLLOWING 2 STAGES OF CYCLIC ACTIVITY COVERING PERIOD FROM 9.00 A.M. UNTIL 5.00 P.M.

Resting stage lasted until 10.45 P.M.

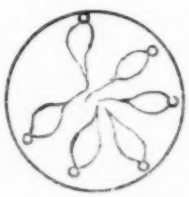

FIG. 9A

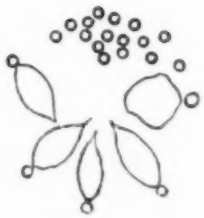

FIG. 9B

FIG. 9A. 10.45 P.M. CELL BECOMES CIRCULAR AND DEVELOPS A DISTINCT WALL

Six oval, highly refractile bodies appear, having a wavy filament on one end and a minute, highly refractile granule on the other. The latter look toward the periphery, the former toward the center. Simulates a protozoal rosette.

FIG. 9B. 11.00 P.M. CELL INCREASES IN SIZE AND WALL BECOMES INDISTINCT

Apparent extrusion of peripherally located granules. Apparent incomplete fusion of 3 oval bodies with resultant gonidial formation.

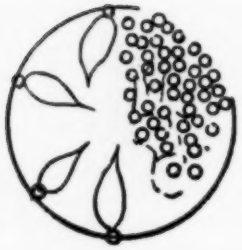

FIG. 10A

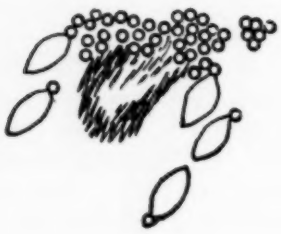

FIG. 10B

FIG. 10A. 11.10 P.M. CELL FURTHER INCREASES IN SIZE

Wall becomes more indistinct, and cannot be made out in the neighborhood of the granules which have increased in number and are actively motile.

FIG. 10B. 11.15 P.M. RUPTURE OF CELL WALL WITH ESCAPE OF GONIDIA

A definite rent appears to three-fourths of depth of cell. Gonidia have rapid oscillatory motion which imparts a movement to the entire cell. Its amplitude of vibration is increased as they are freed into the surrounding medium.

FIG. 11A

FIG. 11B

FIG. 11A. 11.20 P.M. CELL MUCH DIMINISHED IN SIZE, AND OVAL BODIES ARE INDISTINCT

FIG. 11B. 11.30 P.M. THE CELL WALL IS NOW INTACT; CELL IS IRREGULAR IN SHAPE

The oval bodies are imperfectly made out, and little differentiation is present. Cell slowly increases in size until 11.45. It again goes into a resting stage.

Closely related to these forms is another large circular form, usually unstainable, and consequently studied to best advantage in the fresh state. Such forms have been observed under the following conditions: The Berkefeld filtrate of the patient's blood serum developed coccus forms on blood agar, growing readily under aerobic conditions. This culture, after several months' residence in the ice box refused to grow on solid media at 37°. Examination of it microscopically showed what resembled a metachromatic mass of granular debris. When planted at room temperature in plain broth the typical filamentous form of the organism developed, but if planted in broth containing a little hydrocele fluid at 37°, there appeared large coccus forms, which under the warm stage developed cyclic changes similar to the ones just shown. When a drop of this sediment was examined directly in an air tight cell, most remarkable forms were seen. The predominant form is hyaline-like, varies in size from ultramicroscopic to 1 or 5 microns in diameter, and has a clean cut circular appearance. I have observed this form in many cultures under a variety of conditions, and its appearance seems to occur most commonly after apparent fusion and disintegration of the bacterial cells. They are indistinguishable from those adequately described by Hort (1917) in his studies on the meningococcus, although he regards them as ascospores on account of the endosporulation and gemmation and even segmentation which he has observed in them under the warm stage. They certainly give the appearance of reproduction by budding as well as by the schizogony-like process suggested by figure 5. So far, I have not satisfied myself conclusively that gemmation takes place, but I believe it is probable. As Hort has pointed out it is absolutely essential that one study these forms in broth cultures and with wet preparations. To attempt to gain an adequate idea of them with ordinary stains and the use of solid media is to be assured of failure.

I should like to speak of one other morphologic change which is of singular importance regarding the conception of the bacteriology of Vincent's angina and related affections. The symbiosis of *B. fusiformis* and a spirochaete has had traditional

etiologic acceptance. Since 1900, however, there have been a few dissenters, most conspicuous among whom have been Tunnicliff (1905). She contends that the spirilla and *B. fusiformis* are different phases of the same thing. Her principal evidence for the contention lies simply in the fact that in the cultures of *B. fusiformis* certain spiral forms appeared which she feels are identical with the spirochaete of Vincent's angina; but, from her photomicrographs of them as well as from the text, I am inclined to class them among the filamentous and spirillar forms which I have just described. Their width compares favorably with other spirilla and they stain intensely in Loeffler's methylene blue and other simple stains. Such characteristics do not belong to most spirochaetes, including those forms mistaken for such in the ulceromembraneous form of Vincent's angina. These latter stain pink or bluish pink in Romanowsky while both the fusiform and vibrio and spirillar forms of this condition stain a frank blue. We have been able to develop repeatedly in culture the true spirochaete-like forms having the specific staining reaction of the real spirochaete (fig. 12). It goes without saying that they are not genuine spirochaetes; at least I have never observed motility in them. These arise most commonly as lateral or terminal outgrowths from the branching or filamentous form, although more rarely they may spring from the fusiform bacillus itself or they may, under certain conditions, come directly from peripheral chromatin granules of the giant coccus forms. I am not aware that these very slender, wavy forms with the specific staining reaction have ever before been reproduced culturally, from organisms of this class.

Although true spirochaetes may be cultivated from Vincent's angina, as shown by Krumwiede (1913) and others, I do not feel that there is sufficient evidence for regarding them as representative of the majority of the wavy forms found in this condition. Furthermore, by no means all of these forms are motile. There are cultural reasons, as Noguchi (1917) has suggested, for regarding these wavy forms as closely related to the spirilla.

In some experimental subcutaneous and intramuscular ulcers I have observed large numbers of these slender pink-staining

forms, in no way morphologically distinguishable from those of Vincent's angina. They could be seen at times arising from the end of the bacillary form (either diphtheroid or fusiform), and were most numerous when the lesion was nearly healed. It is my contention then, that there are found in these clinical conditions four principal forms: (1) *B. fusiformis;* (2) coarse, sometimes branching, loosely wavy long spiral forms, staining readily; (3) true motile spirochaetes and (4) forms strikingly like spirochaetes in morphology and staining reactions but non-motile, yet easily distinguished from the coarse wavy forms from which they may at times arise, as has been shown in culture. In part from this cultural and experimental evidence, and partly from the fact that in the lesions of the case under discussion spirochaetes were not demonstrated, although fusiform bacilli were cultured from the gangrenous, putrid smelling lesions, and in part for reasons yet to be presented in the study of these organisms, I do not feel that we have adequate evidence for placing a higher etiologic value on the true spirochaetes associated with the fusiforms than we do on the hemolytic streptococci, which may also be associated with them.

GONIDIAL FORMATION

I wish now to present, in brief, evidence tending to show that this strain produces at least three distinct orders of filtrable bodies, and that each form is chiefly, although not wholly derived from a distinct morphologic phase in the cyclic development of the organism. We are in possession of considerable evidence that the granular diphtheroid-like phase develops within itself minute filtrable gonidia, conclusive data regarding the fate of which must be left for future study. The presence of occasional refringent areas located between the staining granules of *B. fusiformis* has been noted by various observers. To a lesser extent these areas are present in diphtheroid bacilli. In the diphtheroid phase of this organism, as well as in other known diphtheroid bacilli, I have observed, under certain conditions, a very marked increase of highly refringent minute circular

bodies, developing in the lighter spaces between the stained bars of the organism. With respect to *B. fusiformis*, it has been stated that the areas are not circular; but, during periods which I wish tentatively to style as gonidio-potent, these spaces seem to be the anlage for the formation of definitely circular bodies.

It is of note that, coincident with a marked intracellular increase of such bodies, very large numbers of similar structures are found free of the bacilli (fig. 13). When growing aerobically, they usually occur in young cultures (six to eight hours) in largest numbers. They appear to stain better as they age, the dye being absorbed first at the poles and on the periphery of the body. They may appear in pairs when free. They may be seen at times on the ends of the organism, and not infrequently bulge from the side, clostridial-like. These considerations suggest strongly the intracellular origin of the extracellular bodies.

Subcutaneous injection of the filtrates into animals has shown results under certain conditions, and more extensive work along this and cultural lines must be done before the nature of these bodies is finally determined. Attempts to grow them have apparently developed forms which appear as very small diplococcoid bodies (stained with Romansky) as well as forms resembling the stellate and giant coccus forms already described. Fig. 14 represents one of the forms, and simulates closely those seen under the warm stage. It is unfortunate that, owing to interruptions in the work, this particular form died out after being laid aside for some months in the ice chest. From their appearance in young cultures, together with their other characteristics, I am inclined to regard these minute bodies as regenerative units, rather than products of degeneration. It seems possible that this process may occur frequently in these organisms, but its extent is greatly influenced by external conditions.

It is not possible at this time to formulate any set of conditions that will with any degree of certainty bring about these periods of gonidia-potent activity. They have occurred under quite diverse and often fortuitous influences apparently, such as the slow evaporation of NaCl solution from a blood agar slant and following transplantation from the depths of an agar shake culture on to a moist blood agar slant.

The French authors, Besson (1913) and Vincent (Besson, 1913), state that these bodies are not spores, inasmuch as they do not have the staining reaction of spores. Tunnicliff (1911) has described spore formation in fusiform bacilli. A personal communication from her indicates that she has based her contention on staining reactions alone, and adds that the bodies may not be true spores. I have noticed a tendency in these granules to retain the stain after decolorization, which has also been shown to be the case with certain diphtheroid bacilli (Mellon, 1917). Although I have made no studies of the tubercle bacillus with respect to the well known granules of Much, the description of the latter inclines me to regard them as comparable to the forms under consideration. These bodies have reacted negatively to the few physiologic tests I have performed with them.

The second order of gonidia is produced chiefly by the filamentous branching forms. They arise either from the end of a main stem or its branches, or directly from the side of either with almost equal frequency (figs. 15 and 2). They stain much deeper than the rest of the cell, and are not resistant to the ordinary stains, as is the first order. Their number depends somewhat on the length of the filament; but, generally speaking, they are more numerous than those of the first order. When a suitable culture was filtered, a finely flocculent precipitate formed in the broth at 37° and at room temperature, which microscopically contained bizarre shaped, irregularly staining bodies, scattered among what was apparently granular debris. Control tubes of this broth did not precipitate, and the nature of this amorphous material cannot be interpreted at present. There is no reasonable doubt concerning the microbic nature of the forms found, as they stain very distinctly; yet, from the fact that they have not yet been successfully transplanted, even though derived from a rapidly growing filament, it is evident that there is much to be learned about them. A variety of forms are discernible. Some are undoubtedly rod forms with rounded ends which are often slightly granular. Others have a diplococcoid appearance, while still others are bizarre shaped,

reminding one of the club-shaped forms of the diphtheria bacillus. It is of interest that none of these forms resemble in the slightest the branching filaments from which they are presumably derived. Fig. 16. Rosenow and Tunnicliff (1913) described small coccus-like bodies in culture from a case very similar to this one. They believe that these coccus forms came from the dilated end of the filaments. They were not able to cultivate them separately, but say they resemble the spores described previously by Tunnicliff. They offer no further suggestions regarding their nature.

The third order of filtrable body I have already described on page 510. They were obtained from the blood serum and also in the third generation of the first blood culture. From the fact that I have not been able to obtain them from any subsequent culture, it is probable that their development in such an early generation is explained by their being carried directly from one culture to another.

Although these orders of gonidia as outlined have developed in connection with these various phases, it is by no means certain that they are always formed in the same way or that bodies answering their description are always seen with the forms described. For example, I have observed occasionally small oval staining bodies in the filamentous forms, and easily staining circular forms on the ends and sides of the granular bacilli. With little regard for size or shape, all spheroidal bodies found in connection with these filamentous forms have received the designation of coccoids. From their difference in behavior it seems probable that there may be a number of bodies of different nature included under this term; and, although it is difficult to speak with assurance regarding all of them, there is a reasonable degree of evidence against all of them being involution or degeneration forms.

CULTURAL AND MORPHOLOGICAL CHARACTERS OF THE DIFFERENT FORMS

The filamentous form occurs with or without branches, varies from 0.5 to 1.5 microns in width, and, in length, from short forms up to those covering one or more fields of the microscope. Under certain conditions large oval knobs may develop at the end of the branches. In common with the filaments, these bodies stain readily and are Gram-negative, and not infrequently contain one or more highly refringent minute bodies, which at times bulge out from their margins. They may occur independently of the filament in which case however, there is usually a short spicule or outgrowth from one or both ends. Optimum growth occurs at room temperature, proceeds slowly in the ice-chest, and may or may not take place in the incubator. The latter temperature seems to conduce to the formation of the terminal oval bodies above mentioned (fig. 6). Beef-heart media seems to be another of the probably large number of factors contributing to the development of these forms.

On slants, growth is luxuriant, the colonies being usually discrete, and after twenty-four hours developing a lemon-yellow pigment on most media. The colonies are raised, moderately moist, fairly adherent, often to the extent of giving the colony a countersunk appearance. In the older colonies, especially, a concentric striation, suggestive of actinomyces, develops. On old, slowly developing slants, coalescence of the colonies is common, and the filaments often fragment into shorter bacillary forms and into diplococci. In transplants the growth is usually filamentous, and it can be said with certainty from warm stage observations that the diplococcus and bacillary forms return directly to the filamentous form on transplantation. In moderately old cultures some of these filaments may lose their staining power and become stippled with fine granules. Similar granules may be seen free of the filaments, and should not be mistaken for gonidial granules, which they resemble closely. Unlike the latter they are not filtrable and are involutionary in nature.

The granular or diphtheroid forms have the characters that are associated with most diphtheroids. They are Gram positive, do not grow perceptibly at room temperature, but in the incubator grow on blood-agar slants, appearing as moist small semitransparent, discrete non-adherent colonies. In the ice-chest, as a rule, growth is not perceptibly increased; but on some media the whole growth may become changed to large coccoid and diplococcoid forms. In broth, a moderate sediment precipitates from a slightly diffuse supernatant, the quantity of which is increased by the presence of serum. Gelatin is not liquefied. This form is a facultative anaerobe.

The diplococcus form, which may also be arranged like staphylococci, grows luxuriantly on any medium, and its optimum temperature is 37°, although it may grow at room temperature. The growth is luxuriant, white, opaque, moist, confluent and non-adherent on solid media, while in liquid media it grows rapidly and diffusely. Funnel-shaped liquefaction of gelatin stabs takes place along the entire line of the inoculation. It has but slight anaerobic tendencies.

In addition to these forms, all of which grow aerobically, is the anaerobic fusiform type, which is to a slight degree a facultative aerobe. On page 507 I have referred to the periplast formation in connection with these forms. Stripped of their periplast, some of these forms would appear as simple vibrios or filaments. I have encountered, in a culture of the latter, large numbers of what at first appeared to be plump, faintly staining bacillary forms; but more careful study showed many of them to be traversed longitudinally by a filament, or else the latter coincided with the margin of the large bacillary-like form (fig. 2). These forms also occurred at the ends of long filaments, giving the impression that they developed there much in the same way as the larger clubs of actinomyces develop. It will be recalled that the latter have the same general arrangement that I have described for these forms, and I think it fair to regard them tentatively as homologues of the actinomyces clubs. The latter, with the exception of those described by Smith (1918) have never been cultivated, and have been regarded

as a degeneration product of the filament, brought about by reaction of the tissues. In this connection it is of interest that these forms developed in the depths of a culture of "hormone agar," the essential feature in its preparation being the preservation of certain growth products of the tissue employed. The forms did not grow in transplants, and have been encountered inconstantly.

No transition changes have been observed with the fusiform organism found here; hence nothing can be postulated definitely in respect to its relation to the filamentous form. However, Tunnicliff's (loc. cit.) contention regarding the phasic relations of *B. fusiformis* and certain spirilla is suggestive in this connection. Moreover, I have recently encountered true branching in a strain of *B. fusiformis*, which more than ever is coming to be regarded as very closely related to the streptothrices.

INDUCED CHANGES OF PHASE

a. Bacillary to filamentous

It is obvious that, in order to establish the theory that these various forms are phases or stages in the life history of a single organism, it will be necessary to trace the mechanism of transformation of one form into the other. A pure culture of the bacillary form, grown on a blood agar slant, was washed down with sterile broth and distributed over another slant of the same media with a Pasteur pipette. After thirty-six hours at 37°, it was put in the ice-box and, after a month, examination showed a tendency to adhere to the medium. Microscopically, the culture showed coccoid bodies, very variable in size, some being as large as 3 microns. Many diplococcoid forms were present, but only a few of the original granular forms could be seen. Such coccoid changes in diphtheroid cultures are among their best known features.

When these coccoids were sown in broth 8, they developed one or more projecting spicules which, in a few days at room temperature, developed into filaments. Morphologically, no indication of this change was observed among the coccoids of the

blood agar slant at this time. The latter was sealed and returned to the ice-chest for six months longer, and at the end of this time the growth seemed still more adherent than before. Microscopically, many of the large coccoid forms and diplococci showed one or more delicate projections, occasionally branched and of variable length (fig. 17). An occasional bacillary form could also be seen with a long terminal filament projecting from its end (fig. 18). Transplants on blood agar of this culture developed the typical branching filamentous forms. They grew best at a temperature slightly above the room, but also developed in the incubator and at room temperature.

A second culture, having precisely the same origin as the above, after remaining in the ice-chest for the same length of time and on the same media, was transplanted on various kinds of media and under varying conditions, in an effort to regain the bacillary form. All attempts over a course of two months were futile, and it seemed reasonable to assume that the culture was dead; but a transplant on blood agar that had stood at room temperature for a month developed the typical filamentous form. The only varying factor in the treatment of these two transplants from an identical source had to do with the fact that the latter was made in the usual way with a wire, while the former was made from a broth suspension, with a pipette. Still a third transplant made with a wire, the origin of which was in common with the two foregoing, refused to develop filaments after one month's stay in the ice-chest when treated in the same manner as the first transplant, but at a later period developed them when treated in a manner entirely different from the other two. These experiments tend to show the obstructions to any precise formulations of a procedure of this kind, theoretical discussion of which will be undertaken presently.

The mechanism for the development of the filaments seems perfectly clear. The demonstration of their origin directly from the bacillus, as well as from its coccoid forms, and their subsequent cultivation as such, seems to rule out both the contamination and the mixed biotype objections. It is noteworthy that thus far it has not been possible to grow the bacillary form

when the culture had changed sufficiently to make transplantation of the filaments possible. In one instance, the change occurred in ten days, yet the bacilli failed to grow in transplant. In control cultures where no such change had taken place, they grew readily, even after much longer intervals.

As I have already noted, the bacillary form has died out, which fact constitutes the only reason why attempts to confirm these results were not made with a pure line, i.e., a culture started from a single bacillus. Yet it is obvious that if gonidia are actually associated with this form, the purity of the line would still be somewhat in doubt. In the many observations that I have made of the pleomorphism of the branching form, I have never observed the large coccoids developing at low temperatures, such as occurred with this diphtheroid form. On the other hand, the filaments almost invariably developed very small diplococci, as involution forms, both in the ice-chest and at room temperature. It is beyond question that slender wavy filaments do develop from diphtheroid forms at times (Mellon, 1915), as well as from *B. fusiformis* (Tunnicliff), but their significance has been an open question, and cannot be considered settled yet. Of interest also in this connection is the demonstration of filaments in the blood of a patient suffering from a severe fusiform infection (gangrenous balanitis) (Mellon, 1919).

b. Coccus to filament

Reference to the blood culture experiments shows the blood serum of the patient was repeatedly filtered through a Berkefeld, and each time, coccus and coccoid forms were obtained. They varied much in size and stained somewhat irregularly, particularly in Gram, where different degrees of reaction to the stain were apparent. Some of the partially decolorized forms contained deeply blue staining granules. Such features in my experience have always suggested that I was not dealing with a stabilized coccus form, but rather with a phase of a higher organism. This culture was repeatedly plated out at 37°, at which temperature it grew luxuriantly; but before a colony was selected

for transplant, it was placed at room temperature for a few days and then in the ice-chest, so as to give opportunity for the development of any mixed forms. This culture was frequently transplanted for ten months on plain agar. It was then planted in an 8-inch tube of no. 8 broth containing some sterile hydrocele fluid. In twenty-four hours, examination of the diffuse growth showed pure cocci. After three days at room temperature, the growth began slowly to change to a flocculent character, at which time the cocci showed a great variation in size, as well as granular staining with slight projections from one or both ends of numerous organisms. At this time, the culture contained numerous non-staining hyaline bodies, some of which had secondary and tertiary circular bodies attached to them, suggesting a budding process. They were best observed when examined in a wet non-stained preparation. Four days later, there were numerous branched filaments in the culture. The original culture of the cocci on plain agar or blood agar continues to grow as such (twenty-four months).

The culture was then transplanted on a solid medium at room temperature in the hope of developing the filaments. After forty-eight hours, nothing but cocci appeared. The culture was then sealed and placed in the ice-box. At the end of four months it showed no macroscopic change, but a microscopic examination gave the appearance of a fusion or degeneration of the coccus forms, poorly defined granules appearing in a non-staining matrix, with Loeffler's blue. Stained in Romanowsky, a great variety of cocci appeared, both as regards size and staining variations. The matrix now stained metachromatically, and was peppered with fine cocci, many of which showed early developing filaments or sprouts (fig. 19). Transplants of this culture on solid media and in broth at 37° gave no growth in a week but, at a temperature between 25° and 30°, developed in three days the typical adherent chromogenic colonies, showing microscopically long branching filaments. Furthermore, all attempts to plate out the coccus form from the filaments have so far been futile.

Inasmuch as neither the filaments nor their involution forms (fine diplococci) were capable of passing my Berkefeld bougies, the only possible explanation of a mixture in this culture seemed to lie in the gonidial granules derived from the filaments. It is, of course, conceivable that they may have been present in the patient's blood. Filtration of the first, as well as distantly removed generations of these cocci, has never yielded a growth of any description, so it would seem that we can dispense with the possibility of a mixture in this instance. However, it should be noted that, of several batches of broth used in these experiments, broth 8 is the only one with which we have been successful in developing the filaments. It is to be remembered that this was the same batch of broth which made possible filamentous development from the blood culture, when other batches gave rise to cocci only. The perfection of synthetic media will, it is hoped, make possible the formulation of conditions, the inconstancy of which has been a formidable source of confusion in studies of this kind.

Associated with these apparently *direct* morphologic transformations, selected because they leave almost nothing to the imagination, are subsidiary ones whose rôle is probably intermediate in nature. Under a variety of conditions and frequently in young cultures, an apparent fusion of the cellular substance of the forms takes place, accompanied by certain staining changes and the development of other forms whose nature is still undemonstrated. These changes simulate what Löhnis and Smith (1916) speak of as symplastic. Almquist (1917) has described similar changes occurring with *B. diphtheriae* and other organisms. They are under further study at present; and, without making any dogmatic assertion regarding their nature, I am sure that the extent and frequency with which they accompany the more direct transformations presages for them a significant rôle in this process.

DISCUSSION

I wish it to be clearly understood that I do not consider these observations as sufficiently complete in themselves to be conclusive proof of the type of cyclic change that I have hypothe-

cated as a working basis. It is obvious that there are many "missing links" in the chain of evidence. Yet the same comment applies in even greater degree if one attempts to explain the observations as a whole in other ways. I am frank to admit that our present knowledge of bacteriological media is not sufficient to enable one to produce some of the reported findings at will; yet it is pertinent that, while certain batches of the media "held out," it was possible to verify them repeatedly.

It is obvious that there are other explanations for the changes described, the commonest, of course, being that the forms are a mixture of different species. Since Barber's invention of a method for the isolation of single bacterial cells, its application in the concrete case has been accepted by many as prerequisite for the demonstration of biologic transformations. It is unquestionably a valuable method in such studies, but, like other single methods, has its limitations, which are more evident to those who have actually used it, perhaps, than to others.

It should be obvious, even to those who sponsor it with a minmum of discrimination, that its usefulness is, roughly speaking, directly proportionate to the size of the organisms which one desires to isolate; and when their size renders them invisible, or nearly so, its categorical quality disappears. It would appear that, to a certain extent at least, these are the conditions prevailing here; and, although the single-cell method is being used where it seems most applicable, its sphere of usefulness can be more precisely delimited only when we learn more of the nature of the filtrable bodies associated with this organism.

There is good reason to believe that otherwise pure cultures of bacteria are often mixtures of biotypes, and such an explanation is usually sufficient in the minds of some to account for *any* experimental transformations. Changes in environment may bring some one of these types into active growth and suppress others, changing very markedly the morphologic picture of a culture.

It is inevitable that this theory of selection should be invoked to explain many phenomena to which it may bear a remote relation, in the same way that Ehrlich's theory of receptors has,

for so many years, retarded advances in immunology. A fair example of such an interpretation is seen in the recent work of Eberson (1918) on the diphtheroid group. The work (Mellon, 1917) which he refutes, is comparable to the subject matter of this paper; yet, approaching it from one angle only, he aligns it with this facile explanation, without even considering the possibility of a cyclic interpretation. In a future communication, it will be possible to focus a more detailed discussion on this point, in the light of this and allied investigations.

It is perfectly obvious that the various phases of this organism are particularly susceptible to changes in environment, yet it should be remembered that the organism itself is not entirely a passive medium. The extent and nature of its response to environmental conditions are functions of its own inherent qualities, which are handed down from generation to generation. However, it is entirely probable that changes may take place in a race of organisms, entirely in accord with their inherent capacity of response to environment, and those changes be so slight as only to become discernible when attempts are made further to modify them in a given direction, which must, of course, be in accord with their capacities for change. Whether we consider such change to occur in a clone, or whether it be the expression of a mixture of biotypes in a culture, bears on our problem only insofar as to require a different combination of environmental factors to evoke the organism's inherent capacities in a certain direction. An instance will point the meaning: reference to the experiments dealing with the development of the filamentous from the bacillary form shows that one cannot always rely precisely on the same means to bring about this result, even though the source of the culture is the same. Even in an organism that has inherent qualities for progressive change, the environmental limits afforded by the usual artificial medium are often inadequate for their evolution. In this instance, the demonstration of filaments arising from the ends of the granular forms leaves little doubt as to their origin.

Furthermore, when cultures become adapted to such conditions, it becomes a matter of increasing difficulty to induce pro-

gressive changes—they become stabilized, so to speak. I have noticed this particularly in attempting to develop the highly refringent intracellular bodies of the granular form; but the filamentous form is more tractable in this respect.

MUTATION

Ordinarily, changes of the sort typified by the bacillary-filamentous transformations would be viewed as mutations, although the whole subject with respect to bacteria seems to lack unanimity of opinion. If we adopt the more conservative idea that mutants only occur under constant conditions, some other interpretation must be forthcoming for these changes, which are to so great a degree a function of environment. Fermentative differences have often been interpreted as evidence of mutation when occurring among the lower bacteria. The literature contains many instances of such in the colon-typhoid group. Hort (1917) has brought to light much evidence for the occurrence of cyclic change in this group, and would in this way explain the so-called mutations. Since reproduction by equal binary fission has always held sway as practically the sole method of bacterial division, the presence of biotypes in cultures could be best explained by mutation; but, if reproduction can occur in other ways, even in a slight degree, the presence of mixed types or variants may have a more simple and satisfactory explanation. The fuso-spirillary organism has the advantage of being on the borderline between the lower and higher bacteria. It is well known that the latter forms have a complex life history, while the so-called branching involution forms of the diphtheria and tubercle bacilli have always suggested close relation with stabilized branching forms.

INVOLUTION AND PLEOMORPHISM: RELATION TO THE WORK OF OTHERS

Finally, it may be considered that this organism represents an example of pleomorphism. This was the explanation advanced for the transformation of certain diphtheroids to diplo-

cocci (Mellon, 1917). Although more marked than the usual pleomorphic changes, they did not seem to merit the designation of the more pretentious process of mutation. Their cyclic nature was suggestive enough to cause this, a similar study, to be approached with those considerations definitely in mind.

Bayon (Besson, 1913) has described an organism from the lesions of leprosy, that has very pleomorphic characters. He says it assumes one of three forms:

> (1) A non-acid-fast and non-acid-resisting streptothrix; (2) a pleomorphic, acid-resisting diphtheroid bacillus, and (3) a definitely acid-fast bacillus, indistinguishable from the bacillus in the tissues.

Williams (Besson, 1913), also grew an organism from leprous lesions, and describes the following forms:

> A, on broth media and on potato-broth, a non-acid-fast streptothrix in the mycelial stage which produced acid-fast rods; B, on milk and lemco-broth, a non-acid-fast diphtheroid bacillus which also produced acid-fast rods; C, on Rost's medium, an acid-fast bacillus which is but the broken-down stage of a streptothrix which was cultivated from a leper passed through respectively all the stages described above.

Dick and Tunnicliff (1918) describe an organism (*Streptothrix putorii*) isolated from a case of rat-bite fever, which resembles this one in many respects. It is noteworthy that they obtained a diplostreptococcus from the blood in the first cultures, which agglutinated with the patient's serum in 1:80, while the streptothrix itself reacted in but 1:20 dilution. We have no way of knowing definitely whether this coccus may have represented a phase in the life history of the organism; and I desire merely to mention it as a possibility, there being no good reason why streptococci might not be present in such cases as well as the additional ones to which I am referring. Middleton (1910) and Douglas, Colebrook and Fleming (1918) have isolated respectively a diplococcus and a *Streptococcus pyogenes* from similar cases, and in the latter instance the organism agglutinated the patient's serum in a 1:60 dilution.

Smith (1918) has recently described a pleomorphic bacillus from the lungs of calves simulating actinomyces. This organism seems to have certain features in common with the fusospirillary organism, chief among which is the cultivation of several distinct morphologic forms which he interprets, not as due to a mixed culture, but as having a cyclic relationship. Certain progressive changes obtain in his culture, as instanced by the complete disappearance of the bacillary form and its replacement by a coccus form. These bacilli, when associated with the club forms, also disappear simultaneously with the development of changes in the latter. Again, they have in common the quality that certain forms resistant to culture are developed from actively growing forms. The development in my cultures of forms that may be homologues of the clubs of actinomyces is another point of contact in the studies. Those of Smith's organism are, of course, real clubs, while the ones I describe are much smaller, but apparently of the same respective origin. The organism described by Lignieres and Spitz (Smith, 1918) and regarded by Smith as practically identical with his organism, develops bacillary forms, which later give rise to diplococci and streptobacilli.

Park and Williams (1910) show pictures of diptheria bacilli, some of which simulate the stellate forms of figure 4. Their description of them betokens the fact that, under the conditions of the experiment, namely, prolonged growth in broth with occasional mechanical disturbance of the pellicle, changes in the reproductive mechanism occur, which suggest to them the process of autogamy and, furthermore that, when the cultures reach this stage, division proceeds *solely* in these involution forms. The fact that similar "branching involution forms" can actually be made to go further and reproduce themselves as branching filaments, is evidence that their observations may admit of a different interpretation in some instances. It would seem that the change produced in the broth by the growth of the organism sooner or later inhibits or prevents development of the organism in its bacillary form; yet its adaptability is expressed in atypical reproductive changes which result in new forms, the latter being the only forms to divide thereafter.

There is no justification at present for saying that all members of the diphtheria group could be made to produce branching filaments, even though the diphtheroid-like phase of this organism in question differs in no essential particular from diphtheroids as we now know them. Still, it should be emphasized that, even as regards the organism with which I am working, it is essential, before the new forms will reproduce as such, that two conditions be fulfilled. The induced changes, typified in part by the above description of Park and Williams, must proceed past a definite point in the evolution of the organism or, on transplantation, the old forms and not the new ones will multiply; secondly, that transplantation must be made to an environment suitable to the newly developing forms, inasmuch as the change in the original medium presumably conditioned their development.

In essence, both the processes of pleomorphism and involution predicate the return of the altered forms to normal on return to "normal conditions." In light of the two considerations just adduced, it repeatedly becomes a question in my mind whether these pleomorphic forms always returned to normal, or whether there occurred a preponderating development of the original forms, the pleomorphic forms either refusing to develop or reproducing so slightly as to be over-looked. The well known selectivity of various media for certain forms would also speak in favor of such a theory.

Furthermore, the apparently lethal effect of residence of cultures in the ice-chest I believe may often be explained in this way: that they refuse to grow, not because they are dead, but because their protoplasm has undergone changes which refuse to respond to the conditions of growth which are not the counterpart of those that have taken place in the organism. I have verified this theory in quite a number of instances when working with organisms of this group; and, without attempting to formulate a rule in accordance with this and associated factors, I may say that temperature changes have been a most potent agency for the induction of evolutionary changes.

RÉSUMÉ

There has been isolated a fuso-spirillary organism from a case in which it caused generalized infection, the point of origin being presumably in the appendix. Its branching filamentous forms relate it closely to the streptothrices, while its bacillary and coccal phases, if we regard them as such, relate it to the lower bacteria. It is noteworthy that the branching filaments were not cultivated from the renal abscess or the lung puncture, although many of them could be demonstrated from the material in both locations—indeed, they constituted the bulk of the flora present in the lung puncture material. They were cultivated from broth blood cultures, however, partly as the result of an irregularity in the preparation of the medium and partly from a radical change in the environmental conditions at a certain stage in the culture's development.

The phenomenon of pleomorphism, undoubtedly well founded, may not explain all morphologic changes in bacteria. Some of those occurring in this strain I am inclined to regard as evolutionary rather than involutionary. As evidence for this view, take for example the demonstration that the filaments originate directly from the body of the granular diphtheroid forms, and can subsequently be cultivated in purity. Furthermore, filtrable forms have been demonstrated in the blood of the patient and in culture, although the ultimate fate of but one order of them has been shown. Warm stage observations have yielded valuable information regarding the origin of some of the filtrable forms, and thrown light on the cyclic activities of the organism. This method is much superior to the single-cell isolation method in a study of this kind, mainly because of the relative invisibility of some of the forms.

I regard the process of phase changes, so to speak, as a function of the environment; but the quality which makes such response possible belongs intrinsically to the organism. Tentatively, at least, I cannot regard these as mutation changes. The latter have been adduced to explain the origin of biotypes from pure lines of bacteria. If one regards mutation conservatively, as of rare occurrence, cyclic changes may offer a better explanation for their origin. Viewed in this light, perhaps some of the

hopeless numbers of bacteria which have received designation as separate species may ultimately come to be considered in some more collective way.

No method can, at present, be accurately formulated for the induction of such changes, not only because there is as yet no such thing as a standard medium, strictly speaking, but even more because of the fact that the stability of bacterial protoplasm is, after all, not absolute. Such lability as it possesses is, however, an intrinsic quality and is not a formidable barrier to the well known quality of cultures to breed true to type—that is, to laboratory type. The transitions as outlined have been repeated many times, but almost never has it been possible to repeat the process in precisely the same way.

Grateful acknowledgment is made to the Directors of this Institution, whose far-sighted policy is responsible for the erection and equipment of an adequate Department of Laboratories, making possible work of an investigative character. I wish also to acknowledge the loan, by the Bausch and Lomb Company, of an apochromatic 2-mm. objective for photographic work, in lieu of one previously purchased but not deliverable until after the war. I wish also to acknowledge the helpful coöperation, clinically, of Dr. S. W. Bradstreet, the physician in charge of the patient on whom the studies were made.

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EXPLANATION OF PHOTOMICROGRAPHS

FIG. 1. Fusiform bacilli grown anaerobically and in pure culture from the renal pus. At *a*, the chromatin has a rod-shaped distribution with a delicate protoplasmic prolongation at either end. Stained in Romanowsky. × 800.

FIG. 2. At *a*, is a form which may represent an homologue of the actinomyces club. Beginning at the left, the deeply stained filament takes a somewhat diagonal course through the periplast, but at the right is marginally located. The faintly staining periplast is divided into three sausage-like loops, the latter of which extends beyond the end of the filament. The remaining two filaments in the field show latterly budding gonidia of varying size. Some larger coccoids are free. Carbol-fuchsin. × 1600.

FIG. 3. The smaller type of giant cocci developing in no. 8 broth blood culture after forty-eight hours. Romanowsky. × 800.

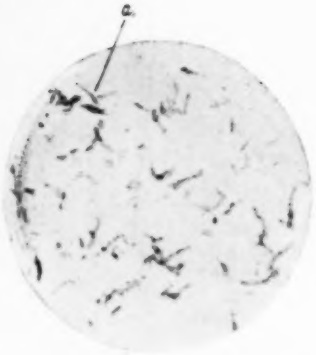
FIG. 4. At *a*, giant coccus forms with peripheral granules one or more of which later give rise to filaments; at *b*, the filaments are further developed. Other smaller coccoid forms of varying size can be seen. These forms have been developed in culture, but represent another type of giant coccus found in No. 8 broth blood culture. Romanowsky stain. × 800.

FIG. 5. In the center of the field is a still larger typ eof giant coccus found in no. 8 broth, blood culture. Four oval intracellular bodies with an early spicule or filament arising from one of them; it crosses the clear sector of the cell at the lower part of the figure. Figure simulates a protozoal schizont. Romanowsky. × 800.

FIG. 6. Filamentous form showing true branching. At *a*, is seen a group of oval spore-like bodies, simulating protozoal flagellates. Romanowsky. × 800.

FIG. 7. In the upper portion is a group of bodies averaging from four to six, peripheral granules. At *a*, early formation of inter-communicating bands between the granules. Many of the latter are free. The lower portion of the figure shows a giant coccus with two small granules on the periphery. Romanowsky. × 1600.

FIG. 8 to 11. Inserted and described in text.



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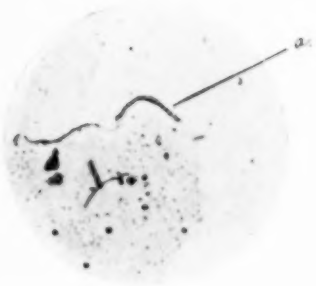

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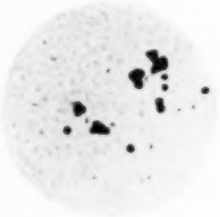

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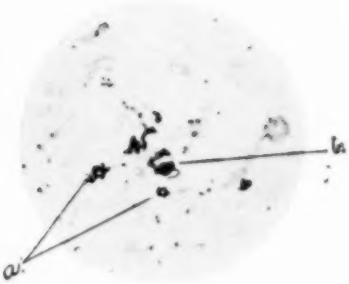

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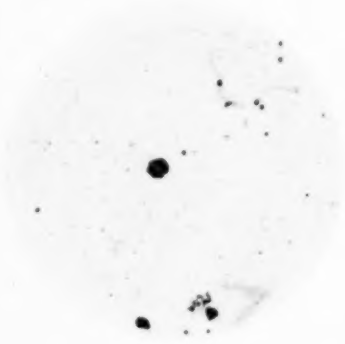

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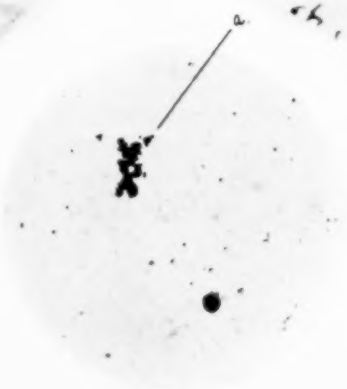

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FIG. 12. Spirochaete-like forms in culture. At *a*, they apparently arise from the bacillary form. Romanowsky. × 800.

FIG. 13. Granular diphtheroid form developing gonidia, many of which are free. At *a*, one is undergoing lateral extrusion. From a twelve-hour blood-agar slant. Specimen overstained in Carbol-fuchsin to bring out the extracellular forms. For this reason little differentiation is seen in the bacillary forms. Carbolfuchsin. × 800.

FIG. 14. The large pear-shaped body in the center of the field developed in a Berkefeld filtrate of the first order of gonidia. It contains several light oval intracellular bodies and two large chromatin granules. Romanowsky. × 1600.

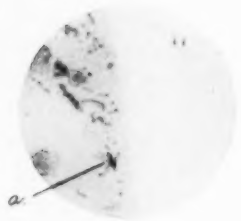
FIG. 15. At *a*, is a filament showing three budding gonidial granules: one is terminal, the other two lateral. This culture was rich in extracellular gonidia a few of which can be seen in this field. The result of a Berkefeld filtrate of this culture is seen in the next figure. Carbol-fuchsin. × 800.

FIG. 16. The more intensely staining pleomorphic forms represent the results of germination of the gonidia shown in figure 15. Carbol-fuchsin. × 800.

FIG. 17. Large coccoid forms derived from the diphtheroid phase showing early development of filaments; at *a*, one develops from a granular bacillus. Carbol-fuchsin. × 800.

FIG. 18. Shows the origin of the filamentous form from a typical diphtheroid form at *a*. Diplococcoids are also present. Carbol-fuchsin. × 2500.

FIG. 19. Early formation of filaments from the diplococcoid phase. Note difference in average size from the coccoids of figure 17. Carbol-fuchsin. × 800.



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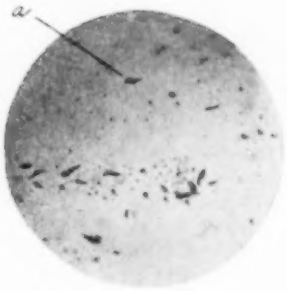

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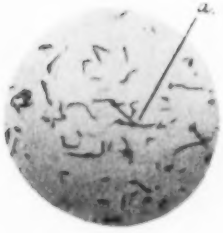

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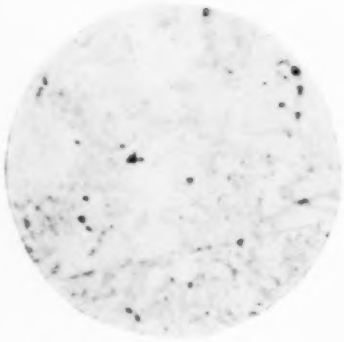

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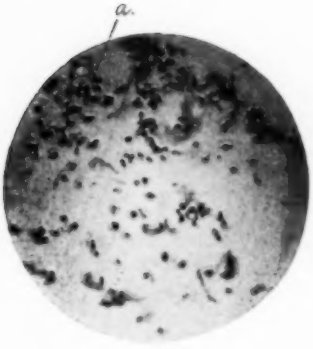

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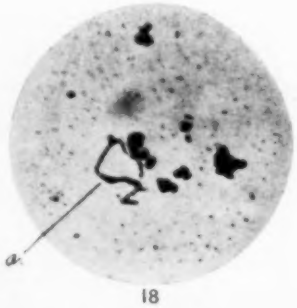

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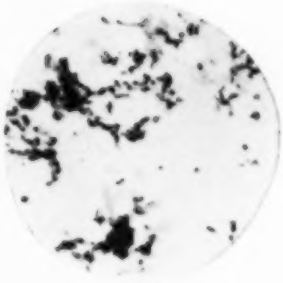

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THE OCCURRENCE OF BACILLUS BOTULINUS IN NATURE

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Van Ermengem, who was the first to isolate *B. botulinus* from poisonous food and who proved that it was the organism which produced the toxin causing the type of food poisoning known as botulism, made repeated efforts to isolate *B. botulinus* from nature. He made cultures of garden soil, dirt from the streets, mud from ponds and rivers, manure from stables, cow manure, horse manure, duck excreta and the intestinal contents of various species of fish. He was never able, however, to find the bacillus except in two specimens of ham which had caused outbreaks of poisoning (Van Ermengem, 1912).

The work of Kemper and Pollack (1897) who recovered a strain from the intestinal contents of a "normal" hog, is the only recorded case of the isolation of *B. botulinus* from nature. This finding, together with the fact that botulism was at that time chiefly connected with poisoning from sausages and ham, led to the belief that *B. botulinus* was a normal inhabitant of the hog intestine.

In 1917 Dr. E. C. Dickson[2] of Stanford Medical School, San Francisco, undertook to check up the work of Kemper and Pollack. Dr. Dickson examined the intestinal contents of 250 grain-fed hogs which were slaughtered for market in South San Francisco. The contents of the colon and ilium were the portions chosen for examination. No trace of *B. botulinus* was

[1] Aided by a grant from the State Council of Defense of California.
[2] Aided by a grant from the State Council of Defense of California.

found in any of the material, showing that *B. botulinus* is not a common inhabitant of the intestines of grain fed hogs.[3]

Dr. Dickson also collected 10 samples of manure from two groups of garbage fed hogs. Cultures were made and examined but no trace of *B. botulinus* was found.

Dr. Dickson collected samples of soil from two gardens where in the summer of 1917 beans were grown and canned, which caused two outbreaks of botulism in January and February, 1918. The specimens were collected in January and February, 1918, and represented a fair sample of the dirt in the yards, both surface and sub-surface, to a depth of 12 inches. *B. botulinus* was not found in any of the samples.

In the summer of 1918, when I made the survey described in the following pages, there were really no positive data as to the occurrence of *B. botulinus* in nature. There were four localities in central California where outbreaks of botulism from home canned fruits and vegetables had occurred during the previous winter, and it seemed that something should be learned by visiting these houses and gardens during the canning season. In collecting the samples, my object was to cover as wide a range of material as possible rather than to make a complete analysis of any one kind. For that reason this report must serve rather as an outline for further research than as a basis for any very definite conclusions.

METHOD OF COLLECTING AND TESTING THE MATERIAL

The samples were collected in sterile test tubes and petri dishes and reached the laboratory within four days. Sterile instruments were used in handling the material in the laboratory. Cultures were made in a double strength beef infusion broth (Van Ermengem's broth) with 2 per cent glucose and a reaction as near neutral as possible (Van Ermengem, 1912). The broth was oil stratified in the tubes. Witte's peptone was used in order to insure reliable toxin production. This broth medium is particularly favorable for the production of botulinus toxin. All the cultures were made in test tubes unless

[3] Dr. Dickson has a report of this work in process of publication.

otherwise stated in the tables. Where flasks were used a large quantity of material was cultured. Just before inoculation all the media were boiled for twenty minutes and immediately cooled by placing the tubes in cold water.

The broth cultures were incubated at 28°C. for from two to six months. They were then tested for toxin. About 10 cc. of the broth culture was passed through a Mandler filter and the filtrate at once oil stratified. One cubic centimeter of this filtrate was injected, subcutaneously, into a guinea-pig. Each filtrate which killed the guinea-pig in forty-eight hours or less, was tested against antitoxins of *B. botulinus* types A and B to determine whether it contained the specific toxin of *B. botulinus*.

For the toxin-antitoxin test three 250 gram guinea-pigs were injected subcutaneously, as follows:

GUINEA PIG NUMBER	FILTRATE	ANTITOXIN
1	1 cc. of filtrate dil. with nor. salt sol. so that 1 cc. kills a 250 gram guinea-pig in about forty-eight hours	1 cc. of antitoxin for *B. botulinus* type A*
2	1 cc. of filtrate dil. with nor. salt sol. so that 1 cc. kills a 250 gram guinea-pig in about forty-eight hours	1 cc. of antitoxin for *B. botulinus* type B
3	1 cc. of filtrate dil. with nor. salt sol. so that 1 cc. kills a 250 gram guinea-pig in about forty-eight hours	Control—no antitoxin

* The antitoxins used were of such strength that 1 cc. neutralized 200 minimum lethal doses of the homologous toxin. One minimum lethal dose of botulinus toxin is that amount which will kill a 250 gram guinea-pig in forty-eight hours.

A reliable toxin-antitoxin test may be obtained with a toxin, 1 cc. of which requires four days to kill. With a weaker toxin the antitoxin test is not reliable for in some cases the pigs develop typical symptoms of botulism but recover after about two weeks while in other cases they remain well for a month and then die with typical symptoms after a few days of illness. In

the following charts four cultures are marked as doubtful because the toxin in the filtrate was too weak to give a decisive toxin-antitoxin test although the symptoms of the guinea-pig that died were entirely typical of botulism.

Where a filtrate gave evidence of containing a weak toxin an effort was made to obtain a stronger toxin by making a transplant from the original culture into a fresh tube of "Van Ermengem's" broth. All the unfiltered portion of the original culture was transferred into the new tube, which was then incubated for about four weeks and tested for toxin. In one case (Palo Alto culture from a bird-pecked and moldy cherry) I was successful in getting from the transplanted culture a strong toxin which proved to be *B. botulinus*, type A.

The incubation period of from two to six months, was too long, as the average life of *B. botulinus* in broth cultures is about three months. My original plan was to incubate for from six to ten weeks. The longer period was necessitated by delays which were unavoidable. I believe it very probable that if the original plan had been adhered to the percentage of positive botulinus cultures might have been higher, for about one-fourth of the guinea-pigs receiving the initial dose of filtrate, died in from two to six weeks, some especially in the Oakdale series, showing marked symptoms of botulism. All efforts to obtain a strong toxin from sub-cultures failed, except in the one case in the Palo Alto series mentioned above.

SURVEY FOR B. BOTULINUS, JUNE, JULY AND AUGUST, 1918

The material was collected from five localities in the central section of California, two of these, Palo Alto and Hollister in the Santa Clara Valley, Oakdale and Madera in the San Joaquin Valley, and Berkeley on San Francisco Bay. The towns were all more than 50 miles distant from each other.

In the Palo Alto series the material had no connection with any previous outbreak of botulism. In the four other localities, however, outbreaks of botulism had occurred during the previous winter, which were caused by home canned fruits and vegetables put up in the summer of 1917. I visited the gardens and the orchard where the vegetables and fruit were grown, except in

Hollister where the peas had been purchased from a peddler. In Hollister and Berkeley an examination was made of the household conditions under which the canning was done. The time chosen for collecting the material corresponded as nearly as possible with the time at which the canning had been done the previous year.

The following is a detailed description of the places and conditions under which the various samples were collected, the character of the samples and the results of the toxin-antitoxin tests. The data are divided into groups according to the places from which the material was collected.

Palo Alto, California

There had been no recorded outbreak of botulism in or near Palo Alto for several years. The corn, beans, peas, peach and cherries which were cultured were obtained in a first class grocery store. All except the cherries were carried direct to the laboratory.

The corn had been set aside as unfit for sale and was badly worm eaten. Four test tube cultures were made of the caterpillars from the corn and five flask cultures of the corn itself. *B. botulinus* was not found in any of the cultures.

One test tube culture each was made of the beans, peas and peach. The peas and beans were dirty and spotted and the peach was partially decayed. The cultures were negative.

The cherries were bought for preserving and were all a little underripe. They were picked over on the unscreened porch of a house in a clean section of the town. There were no horses, cows, or pigs kept anywhere in the neighborhood and dogs were the only animals kept on the immediate place. There were no ants about the house and very few flies. The cherries from which the cultures were made were either slightly bruised or bird pecked. There was no decay or mold on them when they were separated from the other cherries. They were placed in a clean paper bag on the railing of the porch where they remained for two days before they were taken to the laboratory. There

were no spiders or insects in the bag when it was taken to the laboratory but the cherries had molded, wherever the skin was broken.

Nine test tube cultures were made of the cherries. In two of these cultures toxin of *B. botulinus* was produced. One of the positive cultures was made from a bird-pecked moldy cherry, the other from a bruised and moldy cherry. Both contained type A toxin.

Two cultures were made from a caterpillar and four from the crop and gizzard contents of birds killed in the yard of the house where the cherries were picked over. The cultures were all negative.

Hollister, California

The place from which this material was collected was a cross roads saloon about 7 miles from the town of Hollister. It was an exceedingly dirty place. Hogs, cows, and horses were kept in sheds clustering around the back of the house. There was an open privy about 100 feet from the kitchen door. Chickens, mud and manure were everywhere, even on the porches. A leaky water tank was built over a store room adjoining the kitchen. Gray slugs crawled over the walls of the kitchen and store room at night. There were swarms of flies in the house and out of doors.

In the summer of 1917 the housewife canned peas, which she had bought from a peddler. One jar of the peas had spoiled in February, 1918, and the contents was thrown into the garbage which was fed to two hogs. The hogs, one full grown and one small, developed typical symptoms of botulism and *B. botulinus*, type B, was recovered from the stools while the animals were sick. The animals recovered after a few weeks. Three months later, when these specimens were collected, the animals were perfectly healthy, and the small hog had grown to full size. They were later sold to a market.

Cultures were made of the tap water from the kitchen and also of flies collected in the kitchen. In the yard cultures were made from slugs, earth-worms and sowbugs, from chicken

manure, straw and dirt from the chicken yard, from mud under a leaky faucet and from fresh manure collected in the pen of the large hog referred to above.

Toxin of *B. botulinus* type B was produced in one of the five cultures of hog manure. The strain in this culture was of the same type as that isolated from a stool while the hog was sick. It was impossible to make a more complete investigation of the case as the hogs had been killed for market and the pens cleaned up by the time this culture had been tested out. Cultures of all the other material were negative.

Reference has already been made to the work of Kemper and Pollack who isolated a strain of *B. botulinus* from the intestinal contents of a "normal" hog and to the fact that Dr. E. C. Dickson of Stanford Medical School was unable to verify their work although he had examinations made of the intestinal contents of 250 grain-fed hogs and 10 samples of manure from two groups of garbage-fed hogs. The second finding of *B. botulinus*, type B in the manure of the Hollister hog, four months after the contaminated food was eaten suggests very strongly that Kemper and Pollack's "normal" hog may have been fed contaminated food a short time before their cultures were made. Hogs are fairly resistant to botulinus toxin so that there might easily have been no record of the animal's illness.

Oakdale, California

The garden from which material was collected at Oakdale was one in which pole string beans were grown in the summer of 1917. Some of these beans were canned. One of the jars spoiled and was the cause of an outbreak of botulism. In July, 1918, conditions in the garden were practically the same as in the previous year. The section of the town in which this garden was located was quite new. A garden had been made on the place only two years before and no manure had been used. There were no horses, cows, pigs or chickens kept on the place. Beside pole and bush beans, there were tomatoes, wax beans, and melons. The soil was clean sandy loam, well drained. There had been no rain for several months. Irrigating ditches

supplied the water. The samples were collected in the blazing sunlight with the thermometer at 100°F.

Twenty-three cultures were made from spotted leaves and pods of the pole and bush string beans, from ants, spiders, bugs and grasshoppers, collected about the plants and from dirt from the mouth of an ant hill. In one of the three cultures of pole bean leaves spotted by insects or some other small animal toxin of *B. botulinus* type A was produced.

Another culture, containing spiders and small bugs contained a weak toxin. In the toxin antitoxin test the control pig and the pig which received the filtrate botulinus antitoxin type A developed very evident symptoms of botulism about the fourth day but later recovered. As it was impossible to obtain a stronger toxin in transplants from the original, this culture was listed as "doubtful."

Madera, California

The samples were collected from a ranch about 2 miles out of Madera. It was an ordinary small country ranch, with an old house, tank-house, stables and chicken houses. A small orchard of fig and apricot trees adjoined the house on one side and walnut trees bordered the drive and the main road. Alfalfa fields surrounded the place and there were no houses very close by. In 1917 the owners lived on the ranch and kept chickens, turkeys, hogs and horses. There is no record as to whether they also kept cows or as to what sort of a garden they had. In July, 1917, a lot of apricots were canned in a screened summer kitchen. It was windfall fruit and unfit for market. One of the jars of apricots spoiled and caused an outbreak of botulism in which six persons died, February, 1918.

The surviving members of the family moved away and the place was rented to another family. They stayed only a short time. In July, 1918, when I visited the ranch, no one was living there and there were no domestic fowls or animals with the exception of two horses. There were noticeably few birds, bugs or insects other than ants.

One hundred and seventeen cultures were made from material

collected on the ranch. Five cultures were made of tap water from the house. The remaining cultures were made from windfall apricots, which were bird pecked, decayed, moldy or ant eaten; from bugs, flies, ants and spiders; from the beaks, claws and alimentary tract contents of English sparrows, linnets, dove and black birds; from dirt and manure collected in an old hog pen and in the chicken yard where all the chickens had died of botulism six months before; and from fresh horse manure.

No positive culture was found in any of the material. One culture of linnets' claws contained apparently a weak toxin of some sort but it was too weak to give a satisfactory toxin-antitoxin test. The control pig and the filtrate-antitoxin B pig developed typical symptoms of botulism on the third day, but gradually recovered while the filtrate-antitoxin A pig remained normal throughout. This was listed as "doubtful" since it was not possible to obtain a stronger toxin from the transplants.

The ranch at Madera differed from the other localities from which I collected material in that there were no human beings, dogs, cats, pigs, cows, or chickens, living on the place. There was no vegetable garden and very few birds, spiders, insects (other than ants), or other small animals. Neighboring ranch homes were a quarter of a mile distant.

Berkeley, California

In August, 1917, Mrs. M. of Berkeley, California, canned some string beans from her garden. She sterilized them by the fractional method. In January, 1918, it was found that four of the seven jars of beans contained *B. botulinus*. The contents of one jar were fed to some chickens and 24 of them died of botulism, "Limberneck." In August, 1918, Mrs. M. had a few beans growing in her garden in almost the same spot where they had grown the year before and she consented to can these for me, as nearly as she could, in the same manner as those which she had put up the year before. In addition, samples were collected from the house and garden.

The house in which Mrs. M. lived was an old one and was situated in a closely built up neighborhood. The yard was an

ordinary city lot, at the back of which were chicken houses and runs. Between the chicken runs and the house was a garden in which was a small patch of bush string beans and also a patch of mint. The yard was dry at the time my samples were collected. The kitchen and screen porch had been made as clean as possible and there were no flies in either place. Mrs. M. was apparently a woman of average cleanliness.

The beans were gathered from the garden about 1.30 p.m. by Mrs. M. and her daughter, and were picked over by Mrs. M. on the screened porch. She discarded all beans that were broken or decayed. Some of the beans which she passed as good had small hard scars or blemishes on the skin. The beans were washed in several changes of cold water under the faucet in the kitchen. The kettles used to hold the beans were clean. After washing, the beans were cut by Mrs. M. with a clean knife and packed in three Mason quart-jars. Mrs. M. said that she had washed the jars and tops the night before in scalding soap suds. In the morning she had rinsed them in about a dozen changes of cold water. They were again wiped out with a freshly laundered towel just before using. The rubbers were new and were washed in cold tap water from the laundry tub on the screen porch before they were put on the jars.

The jars, with tops loosely screwed, were placed in a deep pot of cold water. The water was brought to a boil. After thirty minutes in the boiling water the jars were removed and the tops screwed down. They were then taken to the laboratory and incubated for six months.

Broth cultures were made from the tap under which the beans were washed and from the tap under which the rubbers were washed. Portions of the beans discarded by Mrs. M. were cultured. Cultures were made of dirt from under the bean plants, of snails, slugs, ants, spiders and a caterpillar, and of beans direct from the garden.

In one of the three jars of beans, canned by Mrs. M. toxin of *B. botulinus* type A was produced, and toxin of *B. botulinus* type B was produced in a culture of a small spider from one of the bean plants. The spider was placed in a sterile tube at

once and did not come in contact with anything in Mrs. M's house. The other material was negative with the exception of two doubtful cultures, one of which was from a sowbug, while the other contained earth, web and droppings from a spider tube in the ground under the bean plants. There was apparently a trace of toxin present in the tubes but it was too weak to give a reliable toxin-antitoxin test.

Forage poisoning

I have recently obtained a culture of *B. botulinus* type A from one of four samples of discolored, moldy hay from a large stack on a ranch near Oakdale, California. This hay was suspected as the cause of one of a series of outbreaks of forage poisoning among horses and mules, occurring in the vicinity of Oakdale in January and February, 1919. The spoilage in the hay ran in veins down through the stack and the samples which were cultured were taken from the deeper layers.

The symptoms exhibited by the horses, as they were described to me by Dr. Eddy of Stockton, California, were very suggestive of botulism. The finding of *B. botulinus* in a sample of the spoiled hay is very suggestive. It does not, however, definitely prove that the animals died of botulism as I was unable to carry out feeding experiments to demonstrate the presence of toxin in the hay.

The successful therapeutic use of a polyvalent botulinus antitoxin in cases of forage poisoning will furnish the best proof of the identity of this poisoning with botulism. An antitoxin furnished by the Agricultural Experiment Station of the University of Illinois was used on two horses of the Oakdale series without success. I have recently tested a sample of this serum and find that it contains only type B antitoxin. In my experience *B. botulinus* type A is more common in California than type B.

Graham and Brueckner, who were the first to claim that forage poisoning was caused by the toxin of *B. botulinus*, have a report of some recent antitoxin work in the Journal of Bacteriology, January, 1919.

SUMMARY

A. Two hundred and thirty-five cultures were made from samples collected in five localities in central California, 50 or more miles distant from each other. The cultures covered a wide range of material, including tap water, hay, leaves, vegetables and fruits in various conditions, insects, spiders, sowbugs, snails, and caterpillars, garden soil, manure from horses, hogs, and chickens, and also samples from the claws and beaks, and crop, gizzard and intestinal contents of birds.

B. Seven cultures containing *B. botulinus* were found. The source of the material in these cultures and the type of toxin demonstrated was as follows:

1. Bruised and moldy cherries.....Palo Alto, Cal., Type A
2. Bird-pecked cherries.....Palo Alto, Cal., Type A
3. Pole bean leaf covered with spots or droppings of insects or small animals.....Oakdale, Cal., Type B
4. Spiders from bush bean plants.....Berkeley, Cal., Type B
5. Bush beans, some of which were slightly scarred, picked over, washed and packed in clean jars for canning.....Berkeley, Cal., Type A
6. Manure from large hog which had recovered from botulism 3 months before sample was taken.....Hollister, Cal., Type B
7. Discolored moldy hay from an outdoor stack..Oakdale, Cal., Type A

Four cultures were found in which there was evidence of toxin but it was so weak that the toxin-antitoxin tests were not considered reliable. The material from which these doubtful cultures were made is as follows:

1. Earth from spider tube, spider droppings and web.....Berkeley, Cal.
2. Sow bug from bush bean plant.....Berkeley, Cal.
3. Linnet claws.....Madera, Cal.
4. Spider and small bugs from bush beans.....Oakdale, Cal.

C. This survey is obviously brief and must serve chiefly as an outline for further research. Nevertheless, the following points seem to be quite clearly indicated.

1. *B. botulinus* is widely distributed in nature.

2. *B. botulinus* is present in the garden and may be on the fruit or vegetables when they are picked.

3. *B. botulinus* is not necessarily associated with active decay. It may be present in the blemishes or spots on the skin of apparently sound fruit and vegetables.

D. The strain of *B. botulinus* found in the hog manure of the Hollister series apparently indicates that *B. botulinus* may remain in the intestinal tract of an animal for at least four months after contaminated food has been eaten.

E. *B. botulinus* may not occur far from the habitation of man. Of the five localities visited, only one failed to give positive results as to the presence of *B. botulinus*. This locality, Madera, differed from the other four in being isolated and deserted. There were no human beings living on the place, no domestic animals other than horses, and there was no vegetable garden.

F. The evidence very strongly suggests that *B. botulinus* may be closely associated with or disseminated by spiders or insects common in gardens in California. Since *B. botulinus* grows readily at temperatures as low as 22°C., there is no reason for assuming that this organism must be associated with the stools of warm blooded animals.

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NOTES ON BACILLUS BOTULINUS

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The production of heterologous toxins by different strains of *B. botulinus* was first recognized by Leuchs, in 1910. He produced antitoxins in horses for both the Ellezelles and Darmstadt strains and found that the antitoxin of the Ellezelles strain would protect against the toxin of its own strain but not against the toxin of the Darmstadt strain, and vice versa.

In 1917 Dr. E. C. Dickson of the Stanford Medical School, San Francisco, produced antitoxins in three goats, which were immunized against his strains III, IV and VI, respectively. He found that strains III and IV, which he had isolated from outbreaks of botulism in California and Oregon were homologous. Strain VI, however, which was isolated in Albany, New York, from cheese which had caused the death of several persons, produced a toxin which was not neutralized by the antitoxin of either strain III or strain IV. Neither would the antitoxin of strain VI neutralize the toxins of strains III or IV.

By means of toxin-antitoxin tests I have identified twelve strains of *B. botulinus* from material obtained in different parts of California and one from an outbreak of food poisoning in Seattle, Washington. Five of the California strains came directly from outbreaks of poisoning from home-canned vegetables and fruits. One came from hay suspected in a case of forage poisoning in horses. The other six had no direct connection with cases of poisoning. (Burke, 1919 b). These twelve strains fall into two distinct types as shown by the toxin-antitoxin

[1] Aided by a grant from the State Council of Defense of California.

experiments. The antitoxin of one type neutralizes the toxin of its own type but not of the heterologous type. I have designated the two as *B. botulinus* Type A and *B. botulinus* type B. The type A strains usually produce a stronger toxin in broth than the type B strains—but aside from this their cultural characteristics on 2 per cent glucose beef infusion agar, double strength beef infusion broth (2 per cent glucose), brain and cooked meat media are identical. There is no difference in morphology.

Dr. Dickson's strains III and IV, to which reference has been made above, are type A strains. The strain which was isolated from cheese by Dr. Nevins in Albany, New York, and which Dr. Dickson refers to as strain VI in his series, is a type B strain. In September 1918 Dr. Robert Graham of the University of Illinois sent me eight cultures containing *B. botulinus* which he had isolated from material from forage poisoning cases among horses and mules in Illinois and Kentucky and from a human case of botulism, traced to canned beans, in Decatur, Illinois. I have tested these cultures against the two types of antitoxin and have found that Dr. Graham's strains are all homologous with my botulinus type B.

It is a point of interest that all of the above nine strains which were isolated in Illinois, Kentucky and New York State should belong to type B, whereas in the Pacific Coast States Dr. Dickson and I have found type A to be predominant. Out of a total of fourteen strains from eight different localities we have isolated nine type A strains and only five type B strains.

PRODUCTION OF BOTULINUS ANTITOXIN FOR LABORATORY PURPOSES

Goats are the best animals to use in producing antitoxin for laboratory tests. As they are very resistant to the toxin of *B. botulinus*, the dosage can be rapidly increased and a good antitoxin produced within a period of two months.

Where goats are not available a weak antitoxin may be produced in rabbits. This can be satisfactorily used in identifying

strains of *B. botulinus* if the toxin is first carefully titrated to determine the minimal lethal dose.[2] I succeeded in carrying two rabbits, one for type A and one for type B toxin, through the eighth injection. The initial dose was about one-fourth of a minimum lethal dose. The dose was increased very gradually each week. At the eighth week each rabbit received 10 minimum lethal doses. After this injection one of the animals developed severe symptoms of botulism and it was thought unwise to carry the injections further as five rabbits had already been lost. It was found that the serum of each rabbit contained a weak antitoxin which would neutralize from six to ten minimum lethal doses of the homologous toxin.

TECHNIQUE FOR ISOLATING B. BOTULINUS

B. botulinus may be readily isolated from agar transplants made direct from the contents of cans of spoiled food because there are, as a rule, few other bacteria present.[3] When however, materials such as manure and intestinal contents are to be tested, it is difficult to recover the organism from the original agar transplants because material of this sort is rich in strict anaerobes and facultative anaerobes and the number of botulinus bacilli is comparatively small.

The most reliable technique is to inoculate from the original material into cultures of Van Ermengem's broth[4] and chopped meat media.[5]

[2] One minimum lethal dose of botulinus toxin is that amount which will kill a 250 gram guinea-pig in forty-eight hours.

[3] In one case I have isolated *B. botulinus* from a jar of beans which showed signs of spoilage but in which no toxin could be demonstrated by animal inoculation. The strain was a typical type B strain, producing a moderately strong toxin in Van Ermengem broth culture. This demonstrates the importance of making cultural as well as toxin tests on canned food sent in to the laboratory for examination.

[4] 1000 grams of lean beef to the liter, 5 grams NaCl, 10 grams Witte's peptone, 2 per cent glucose. The medium oil stratified in the tubes. (See Van Ermemgem, E. 1912.)

[5] Finely chopped beef heart or lean meat is mixed with two volumes of water and brought slowly to the boil, being stirred constantly. It is neutralized, tubed and autoclaved at 15 pounds pressure for 30 minutes.

The broth and meat cultures are richly inoculated with the suspected material. Duplicate cultures are made, one set being heated to 60°C. for one hour to destroy the non-spore bearing organisms. The cultures are then incubated at 28°C. after which portions are filtered and tested for toxin by injecting 1 cc. of the filtrate subcutaneously into a 250 gram guinea-pig. If the pig dies within four days the filtrate is tested against the antitoxins of *B. botulinus* types A and B to determine the presence of specific botulinus toxin. For this purpose the filtrate is diluted with saline so that 1 cc. will kill a 250 gram guinea-pig in approximately forty-eight hours. Three 250 gram guinea-pigs are selected. The first pig receives 1 cc. of diluted filtrate mixed with antitoxin for *B. botulinus* type A. The second pig receives 1 cc. of the diluted filtrate mixed with antitoxin of *B. botulinus* type B. The third pig is the control and receives 1 cc. of the diluted filtrate but no antitoxin. Pure cultures of *B. botulinus* type B produce usually a little more than one minimal lethal dose of toxin per cubic centimeter after three days growth in meat or Van Ermengem broth medium. Type A strains produce a stronger toxin in the same time. In mixed cultures, however, the toxin production may be much delayed and a negative diagnosis should not be made until the cultures have been incubated for three weeks.

The organism is isolated from the broth or meat culture in which specific toxin is demonstrated. In place of the usual anaerobic plating methods it is the practice in this laboratory to use thinly seeded agar shake cultures. After incubation these cultures are examined and fished by transmitted light on the stage of a dissecting microscope such as is used in Zoological laboratories. A lens magnifying 10–20 diameters is most satisfactory. (See E. C. Dickson and G. S. Burke, 1918.)

The development of this technique was necessary because the hydrogen plating methods used at first were too slow to permit of the examination of large amounts of material. Shake cultures are easily and quickly made and there is little chance for contamination. The magnification of 10 to 20 diameters afforded by the dissecting microscope lens gives a clear and detailed

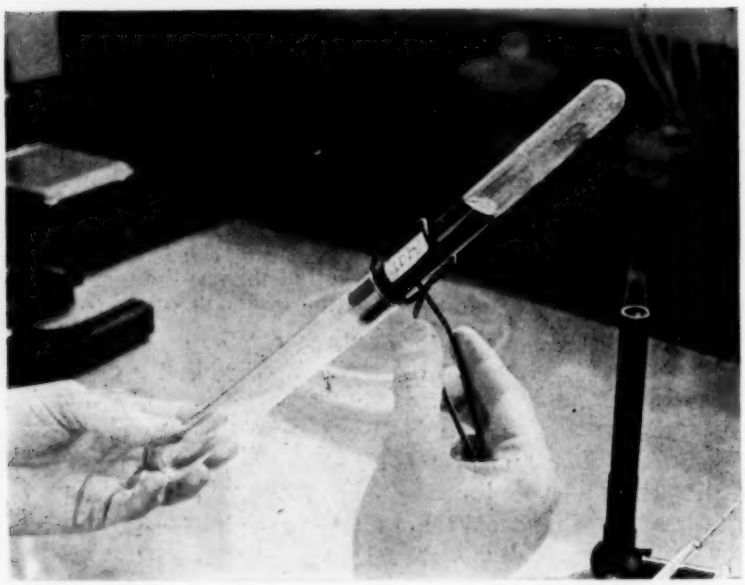
colony picture which is much more satisfactory for the observation of deep agar colonies than that given by the compound microscope. The method has proven so accurate and economical both of time and materials that it seems worth while to give a more detailed description than was given in the former paper.

In detail the technique is as follows. A series of agar shake cultures (5–8) are made from the contaminated Van Ermengem broth cultures, jars of food, etc. (Beef infusion agar, plus 2 per cent glucose, is used, with a reaction as near neutral as possible.) Just before the inoculation is made the agar is boiled for twenty minutes and cooled to approximately 47°C. It is essential to the success of the technique that the agar cultures be thinly seeded in order that the colonies shall be of good size and well separated. Successive dilutions may be used as in plating. As soon as the agar tube is inoculated it is thoroughly shaken and plunged into cold water to harden, after which it is incubated. One very good method of mixing the material in deep agar tubes is to hold the tube in a vertical position grasping it by its upper end. By twisting the wrist the lower end of the tube is then rotated about the vertical axis while the upper end remains practically stationary.

Botulinus colonies may appear in twenty-four hours at 37½°C. or in thirty-six hours at 28°C. Under some circumstances they require a much longer time. (Burke, 1919a.) In mixed cultures the presence of antagonistic organisms may inhibit the growth. One set of agar cultures should be incubated at 37½°C. and another at 28°C., since *B. botulinus* grows almost equally well at either temperature while some of the contaminating organisms are distinctly inhibited by one or the other temperature. After incubation the colonies in the agar tube are observed and fished under the dissecting microscope by transmitted light.

There are two methods of preparing the agar culture for fishing. In the first, the cotton plug is removed from the culture tube and the tube inverted, with the open end pointed downward into a sterile tube of greater diameter than that of the culture tube. The closed end of the agar culture tube is then passed slowly into the flame of a bunsen burner. The expansion,

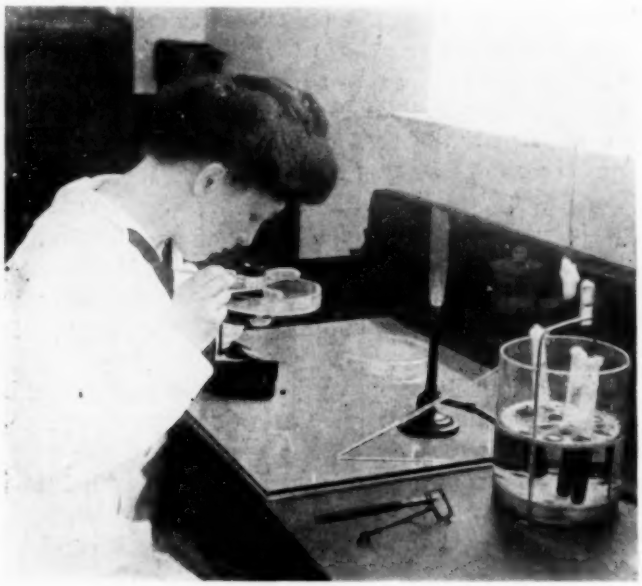
caused by the heat, drives the agar column out of the culture tube into the large tube, from which it is emptied into a petri dish for fishing. In the second method a sharp file mark is made around the culture tube about one-half inch from the bottom. The tube is then placed in a large petri dish and broken by applying a piece of red hot glass rod to the file mark. By driving the cotton plug down into the culture tube, the agar



column is pushed out of the broken end so as to expose any desired colony.

The colony chosen for fishing is placed in position under the dissecting microscope lens. The surface of the agar above the colony is sterilized by brushing a red hot platinum loop very lightly over it. The agar should be slightly melted by this operation, giving a smooth glossy surface through which a clear image of the colony can be had. A short platinum needle (1 to $1\frac{1}{4}$ inches) is used for fishing. This is sterilized and plunged down through the sterile agar surface into the colony.

Agar shake cultures are made from the material fished from the colonies. These transplants are as a rule thinly seeded. If they are not pure it is an easy matter to find in them well isolated colonies which can be successfully fished without contamination. Final identification of the isolated organism is made by culturing it in broth or meat and testing the filtrate for specific botulinus toxin.



COLONY CHARACTERISTICS OF B. BOTULINUS

Deep agar colonies of *B. botulinus*, as seen under the dissecting microscope with a magnification of 10 to 20 diameters are very characteristic. The description here given is based upon a study of nine pure strains isolated at Stanford University. Seven of the strains were isolated from mixed cultures on the basis of these colony characteristics.

In a young culture the typical Botulinus colony (see plates 1 and 2) is a thin translucent disc, having a small opaque knot or "nucleus" on or near the periphery at one side. The edge of the disc is either flattened or sharply indented at the point nearest the knot. The disc is more or less completely filled with clear spots or "vacuoles" which are in reality gas bubbles caught in the meshes of the bacterial growth as shown by examination of stained smears of the colonies. The outline of the colony is always clear cut and definite.

The translucent appearance of the colonies is due to the comparative thinness of the disc and to the presence of the gas bubbles. The gas bubbles may be of any size. Sometimes they are so large that seven or eight of them fill the disc. No photograph was obtained of such a colony. Again they may be so small as to look like little white specks. The gas bubbles when visible are diagnostic of *Bacillus botulinus*. I have been unable to find any other anaerobe producing this type of colony, although I have examined more than 400 cultures from material rich in anaerobes, and also cultures of the common pathogenic anaerobes.

The knot or "nucleus" in a young culture is very small and dense. In old cultures it develops short stubby processes, which are nevertheless translucent. The diameter of the tuft thus formed, however, is never greater than the diameter of the disc.[6] One of my strains rarely shows any "nucleus" or irregularity of outline, but its gas bubbles are very distinct.

Another very typical feature of botulinus colonies is the transparency of the disc in old cultures after three to six weeks incubation. See plate 3, figures 1 and 2.

In plate 2, figure 3, a very small colony can be seen at the edge of the large colony. The disc is comparatively small and

[6] The pictures of botulinus colonies given by V. Hibler (1908) are similar to the colonies of certain anaerobes which I have encountered as contaminants in my work with *B. botulinus*. Colonies of that type with a heavy hairy tuft at one side of the disc are not found in pure cultures of the strains of *B. botulinus* which we have in this laboratory.

has the appearance of a clear gas bubble. This suggests that the opaque knot is the original nucleus of growth and that as gas is produced the agar is split and the bacteria grow into the space thus formed. The agar is sometimes split in more than one plane (plate 1 and plate 3, figs. 4 and 5).

The colony characteristics of *B. botulinus* are the same on either extract or infusion agar and the reaction of the medium does not affect them. The variations which occur are apparently due to a difference in the stiffness of the medium or to the size of the colonies. In very large colonies it is difficult and sometimes impossible to see the gas bubbles.

GENERAL CULTURAL CHARACTERISTICS OF B. BOTULINUS

The optimum temperature for *B. botulinus* as originally recorded was 22°–28°C. The pure strains isolated at Stanford, however, grow somewhat more rapidly at 37½°C. than at 28°C. At 37½°C. agar shake cultures show well defined and typical growth in 24 hours. At 28°C. 36–40 hours are required for the same growth, and at lower temperatures the rate of development is still slower. Toxin is produced at 37½°C. as well as at 28°C.

B. botulinus is a proteolytic anaerobe. It digests and darkens but does not blacken meat and brain media. It produces strong toxin in meat cultures and the organisms remain viable for a much longer period in meat and brain cultures than in broth or agar.

In Van Ermengem broth cultures after 40 hours incubation at 28°C. *B. botulinus* produces a delicate clouding of the medium. If the culture is not shaken the broth just under the oil is usually found to be clear. After incubating for a month or more the cloud disappears leaving the broth clear. In a pure culture there is no noticeable sediment in either the young or old cultures. Other observers have described a heavy sediment in old cultures but in my experience a heavy sediment is a sign of contamination.

My strains of *B. botulinus* do not spore readily in either broth

or agar. In brain media spores sometimes appear in two weeks but usually it requires a much longer period.

In infusion agar, 2 per cent glucose, the anaerobe line for *B. botulinus* is one centimeter below the surface. When extract agar is used, however, the anaerobe line drops to two centimeters. For this reason extract agar tubes should contain at least three inches of agar.

SUMMARY

1. There are two known types of *B. botulinus*, which are here designated type A and type B. They produce heterologous toxins and are easily identified by a toxin-antitoxin test.

2. Antitoxin for laboratory purposes may be produced from either goats or rabbits. Goats are the more satisfactory animals.

3. The most reliable method of demonstrating the presence of *B. botulinus* in contaminated material is to make Van Ermengem broth or meat cultures, incubate for three weeks, filter and test the filtrate for specific toxin.

4. The use of agar shake cultures, examined and fished under a dissecting microscope, is a simple and reliable technique for the isolation of *B. botulinus* from contaminated material or cultures.

5. *B. botulinus* produces colonies in agar which are characteristic and can be readily identified.

6. The strains of *B. botulinus* isolated at Stanford University grow and produce toxin as readily at 37½°C. as at 28°C. They do not produce a heavy sediment in broth and they do not produce spores readily in either broth or agar.

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PLATE 1

Fig. 1. Deep agar colony of *B. botulinus* from forty-eight hour culture. *d* indicates the edge of a small disc lying in a different plane from the large disc.

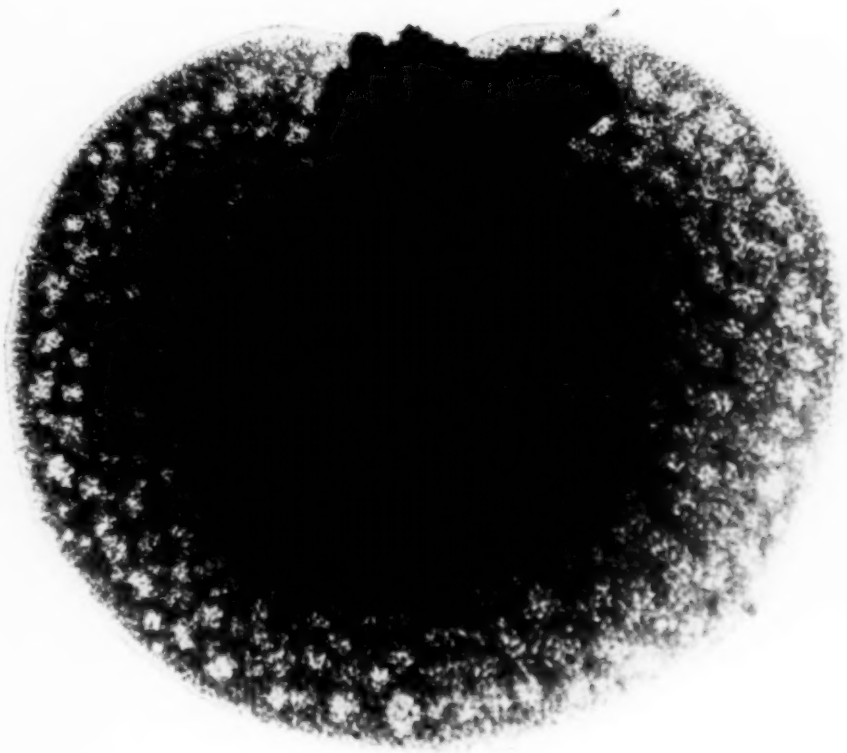
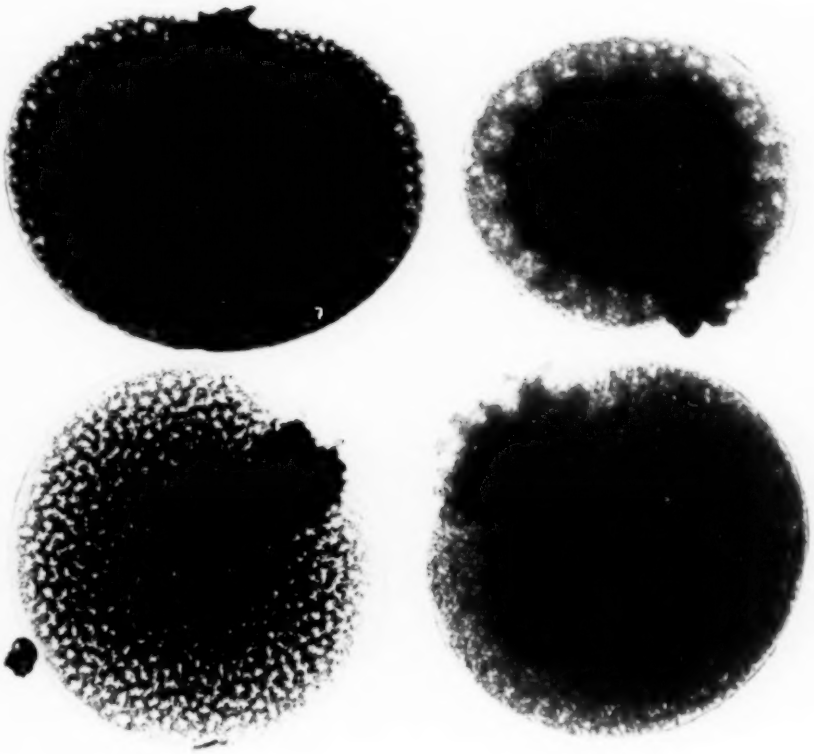


PLATE 2

Figs. 1 and 2. Deep agar colonies of *B. botulinus* from forty-eight hour culture, showing varying size of gas bubbles.

Fig. 3. Deep agar colony of *B. botulinus* from a week old culture, with a very small colony lying near the edge of the large colony.

Fig. 4. Deep agar colony of *B. botulinus* from a week old culture, showing minute gas bubbles in the disc, and branching processes of the "nucleus."



(Burke: Notes on *Bacillus botulinus*)

PLATE 3

Figs. 1 and 2. Deep agar colonies of *B. botulinus* from four weeks old culture, showing transparency of disc and branching processes of the "nucleus."

Fig. 3. Deep agar colony of *B. botulinus* from a three weeks old culture. View of the edge of colony.

Figs. 4 and 5. "Composite colonies" of *B. botulinus*, having three discs lying in different planes.

Figs. 6 and 7. Two deep agar colonies from the same week old agar culture. Figure 6 shows the minute gas bubbles in the disc and the branching processes of the "nucleus." In figure 7 the growth in the disc of the colony has diffused into the surrounding medium.

(Burke: Notes on *Bacillus botulinus*)